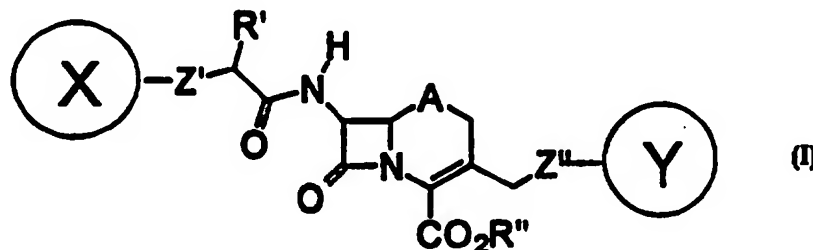




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q		A2	(11) International Publication Number: WO 96/30540
			(43) International Publication Date: 3 October 1996 (03.10.96)
(21) International Application Number: PCT/US96/04059		(74) Agent: STORELLA, John, R.; Townsend and Townsend and Crew, Steuart Street Tower, 20th floor, One Market Plaza, San Francisco, CA 94105 (US).	
(22) International Filing Date: 20 March 1996 (20.03.96)			
(30) Priority Data: 08/407,544 20 March 1995 (20.03.95) US		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(60) Parent Application or Grant (63) Related by Continuation US 08/407,544 (CIP) Filed on 20 March 1995 (20.03.95)			
(71) Applicant (for all designated States except US): THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612 (US).		Published Without international search report and to be republished upon receipt of that report.	
(72) Inventors; and (75) Inventors/Applicants (for US only): TSIEN, Roger, Y. [US/US]; 8535 Nottingham Place, La Jolla, CA 92037 (US). ZLOKARNIK, Gregor [DE/US]; 3329 Jemez Drive, San Diego, CA 92117 (US).			

(54) Title: SUBSTRATES FOR BETA-LACTAMASE AND USES THEREOF



(57) Abstract

Substrates for β -lactamase of general formula (I) in which one of X and Y is a fluorescent donor moiety and the other is a quencher (which may or may not re-emit); R' is selected from the group consisting of H, lower (i.e., alkyl of 1 to about 5 carbon atoms) and $(\text{CH}_2)_n\text{OH}$, in which n is 0 or an integer from 1 to 5; R'' is selected from the group consisting of H, physiologically acceptable metal and ammonium cations, $-\text{CHR}^2\text{OCO}(\text{CH}_2)_n\text{CH}_3$, $-\text{CHR}^2\text{OCOC}(\text{CH}_3)_3$, acylthiomethyl, acyloxy-alpha-benzyl, delta-butyrolactonyl, methoxycarbonyloxymethyl, phenyl, methylsulphonylmethyl, beta-morpholinoethyl, dialkylaminoethyl, acyloxyalkyl, dialkylaminocarbonyloxymethyl and aliphatic, in which R² is selected from the group consisting of H and lower alkyl; A is selected from the group consisting of S, O, SO, SO₂ and CH₂; and Z' and Z'' are linkers for the fluorescent donor and quencher moieties. Methods of assaying β -lactamase activity and monitoring expression in systems using β -lactamase as a reporter gene also are disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

5 SUBSTRATES FOR BETA-LACTAMASE AND
 USES THEREOF

 BACKGROUND OF THE INVENTION

 The present invention relates generally to the
10 fields of chemistry and biology. More particularly, the
 present invention relates to compositions and methods for use
 in measuring gene expression.

 A reporter gene assay measures the activity of a
 gene's promoter. It takes advantage of molecular biology
15 techniques, which allow one to put heterologous genes under
 the control of any promoter and introduce the construct into
 the genome of a mammalian cell [Gorman, C.M. et al., *Mol. Cell*
 Biol. 2: 1044-1051 (1982); Alam, J. and Cook, J.L.,
 Anal.Biochem. 188: 245-254, (1990)]. Activation of the
20 promoter induces the reporter gene as well as or instead of
 the endogenous gene. By design the reporter gene codes for a
 protein that can easily be detected and measured. Commonly it
 is an enzyme that converts a commercially available substrate
 into a product. This conversion is conveniently followed by
25 either chromatography or direct optical measurement and allows
 for the quantification of the amount of enzyme produced.

 Reporter genes are commercially available on a
 variety of plasmids for the study of gene regulation in a
 large variety of organisms [Alam and Cook, *supra*]. Promoters
30 of interest can be inserted into multiple cloning sites
 provided for this purpose in front of the reporter gene on the
 plasmid [Rosenthal, N., *Methods Enzymol.* 152: 704-720 (1987);
 Shiau, A. and Smith, J.M., *Gene* 67: 295-299 (1988)]. Standard
 techniques are used to introduce these genes into a cell type
35 or whole organism [e.g., as described in Sambrook, J.,
 Fritsch, E.F. and Maniatis, T. Expression of cloned genes in
 cultured mammalian cells. In: *Molecular Cloning*, edited by
 Nolan, C. New York: Cold Spring Harbor Laboratory Press,

1989]. Resistance markers provided on the plasmid can then be used to select for successfully transfected cells.

Ease of use and the large signal amplification make this technique increasingly popular in the study of gene regulation. Every step in the cascade *DNA --> RNA --> Enzyme --> Product --> Signal* amplifies the next one in the sequence. The further down in the cascade one measures, the more signal one obtains.

In an ideal reporter gene assay, the reporter gene under the control of the promoter of interest is transfected into cells, either transiently or stably. Receptor activation leads to a change in enzyme levels via transcriptional and translational events. The amount of enzyme present can be measured via its enzymatic action on a substrate. The substrate is a small uncharged molecule that, when added to the extracellular solution, can penetrate the plasma membrane to encounter the enzyme. A charged molecule can also be employed, but the charges need to be masked by groups that will be cleaved by endogenous cellular enzymes (e.g., esters cleaved by cytoplasmic esterases).

For a variety of reasons, the use of substrates which exhibit changes in their fluorescence spectra upon interaction with an enzyme are particularly desirable. In some assays, the fluorogenic substrate is converted to a fluorescent product. Alternatively, the fluorescent substrate changes fluorescence properties upon conversion at the reporter enzyme. The product should be very fluorescent to obtain maximal signal, and very polar, to stay trapped inside the cell.

To achieve the highest possible sensitivity in a reporter assay one has to maximize the amount of signal generated by a single reporter enzyme. An optimal enzyme will convert 10^5 substrate molecules per second under saturating conditions [Stryer, L. Introduction to enzymes. In: *Biochemistry*, New York: W. H. Freeman and company, 1981, pp. 103-134]. β -Lactamases will cleave about 10^3 molecules of their favorite substrates per second [Chang, Y.H. et al., *Proc.Natl.Acad.Sci.USA* 87: 2823-2827 (1990)]. Using a

fluorogenic substrate one can obtain up to 10^6 photons per fluorescent product produced, depending on the type of dye used, when exciting with light of the appropriate wavelength. The signal terminates with the bleaching of the fluorophore

5 [Tsien, R.Y. and Waggoner, A.S. Fluorophores for confocal microscopy: Photophysics and photochemistry. In: *Handbook of Biological Confocal Microscopy*, edited by Pawley, J.B. Plenum Publishing Corporation, 1990, pp. 169-178]. These numbers illustrate the theoretical magnitude of signal obtainable in

10 this type of measurement. In practice a minute fraction of the photons generated will be detected, but this holds true for fluorescence, bioluminescence or chemiluminescence. A good fluorogenic substrate for a reporter enzyme has to have a high turnover at the enzyme in addition to good optical

15 properties such as high extinction and high fluorescence quantum yield.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide

20 β -lactamase substrate compounds. It is a further object of the invention to provide membrane-permeant compounds. The membrane-permeant compounds may be transformed into substantially membrane-impermeant compounds.

Another object of the invention is to provide β -

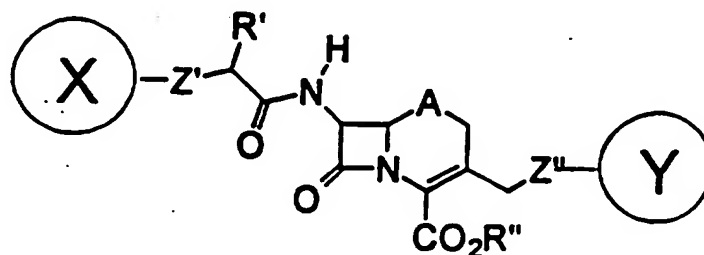
25 lactamase reporter genes. A further object of the present invention is to create cells containing the β -lactamase reporter genes functionally linked to a promotor such that when the promotor is turned on, the reporter gene will be expressed. Expression of the β -lactamase is measured with the

30 β -lactamase substrates which emit light after hydrolysis by the β -lactamase.

A further object of the invention is to use the β -lactamase reporter genes in cells and the β -lactamase substrate compounds of the present invention to screen for

35 biochemical activity.

In accordance with the present invention, fluorogenic substrates are provided of the general formula I



wherein:

one of X and Y is a fluorescent donor moiety or a membrane-permeant derivative thereof, and the other is a quencher moiety, an acceptor fluorophore moiety or a membrane-permeant derivative thereof;

R' is selected from the group consisting of H, lower alkyl, $(\text{CH}_2)_n\text{OH}$, $(\text{CH}_2)_n\text{COOR}''$, and $=\text{NOJ}$, in which n is 0 or an integer from 1 to 5 and J is H, Me, CH_2COOH , CHMeCOOH , and CMe_2COOH ;

R'' is selected from the group consisting of H, physiologically acceptable metal and ammonium cations, $-\text{CHR}^2\text{OCO}(\text{CH}_2)_n\text{CH}_3$, $-\text{CHR}^2\text{OCOC}(\text{CH}_3)_3$, acylthiomethyl, acyloxy-alpha-benzyl, delta-butyrolactonyl, methoxycarbonyloxymethyl, phenyl, methylsulphinylmethyl, betamorpholinoethyl, dialkylaminoethyl, dialkylaminocarbonyloxymethyl, in which R² is selected from the group consisting of H and lower alkyl;

A is selected from the group consisting of S, O, SO, SO₂ and CH₂;

Z' is a linker for X; and

Z'' is a linker for Y.

In another aspect, this invention provides methods for determining whether a sample contains β -lactamase activity. The methods involve contacting the sample with a compound substrate of the invention, which exhibits fluorescence resonance energy transfer when the compound is excited; exciting the compound; and determining the degree of fluorescence resonance energy transfer in the sample. A

degree of fluorescence resonance energy transfer that is lower than an expected amount indicates the presence of β -lactamase activity. One embodiment of this method is for determining the amount of an enzyme in a sample. According to this method, determining the degree of fluorescence resonance energy transfer in the sample comprises determining the degree at a first and second time after contacting the sample with the substrate, and determining the difference in the degree of fluorescence resonance energy transfer. The difference in the degree of fluorescence resonance energy transfer reflects the amount of enzyme in the sample.

In another aspect, this invention provides recombinant nucleic acid molecule comprising expression control sequences adapted for function in a vertebrate cell and operably linked to a nucleotide sequence coding for the expression of a β -lactamase. It also provides recombinant nucleic acid molecules comprising expression control sequences adapted for function in a eukaryotic cell and operably linked to a nucleotide sequence coding for the expression of a cytosolic β -lactamase. In certain embodiments, the invention is directed to mammalian host cells transfected with these recombinant nucleic acid molecules.

In another aspect, this invention provides methods for determining the amount of β -lactamase activity in a cell. The methods involve providing a host cell transfected with a recombinant nucleic acid molecule comprising expression control sequences operatively linked to nucleic acid sequences coding for the expression of a β -lactamase; contacting a sample comprising the cell or an extract of the cell with a substrate for β -lactamase; and determining the amount of substrate cleaved, whereby the amount of substrate cleaved is related to the amount of β -lactamase activity.

In another aspect, this invention provides methods for monitoring the expression of a gene operably linked to a set of expression control sequences. The methods involve providing a host cell transfected with a recombinant nucleic acid molecule comprising expression control sequences operatively linked to nucleic acid sequences coding for the

expression of a β -lactamase, except if the eukaryote is a fungus, wherein the β -lactamase is a cytosolic β -lactamase; contacting a sample comprising the cell or an extract of the cell or conditioned medium with a substrate for β -lactamase; and determining the amount of substrate cleaved. The amount of substrate cleaved is related to the amount of β -lactamase activity.

In another aspect, this invention provides methods for determining whether a test compound alters the expression of a gene operably linked to a set of expression control sequences. The methods involve providing a cell transfected with a recombinant nucleic acid construct comprising the expression control sequences operably linked to nucleic acid sequences coding for the expression of a β -lactamase except if the eukaryote is a fungus, wherein the β -lactamase is a cytosolic β -lactamase; contacting the cell with the test compound; contacting a sample comprising the cell or an extract of the cell with a β -lactamase substrate; and determining the amount of substrate cleaved, whereby the amount of substrate cleaved is related to the amount of β -lactamase activity. In one embodiment of the methods, the substrate is a compound of this invention. The step of determining the amount of substrate cleaved comprises exciting the compound; and determining the degree of fluorescence resonance energy transfer in the sample. A degree of fluorescence resonance energy transfer that is lower than an expected amount indicates the presence of β -lactamase activity.

In another aspect, this invention provides methods of clonal selection comprising providing cells transfected with a recombinant nucleic acid molecule comprising the expression control sequences operably linked to nucleic acid sequences coding for the expression of a cytosolic β -lactamase; contacting the cells with a substance that activates or inhibits the activation of the expression control sequences; contacting the cells with a compound of claim 9 which is converted into a substrate; determining whether substrate is cleaved within each individual cell, whereby

cleavage reflects β -lactamase activity; selecting and propagating those cells with a selected level of β -lactamase activity. In a further embodiment, the method further involves culturing selected cells in the absence of activator for a time sufficient for cleaved substrate to be substantially lost from the cells and for β -lactamase levels to return to unactivated levels; incubating the selected cells with a compound of claim 9 which is converted into a substrate; and selecting cells that have not substantially cleaved the substrate.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1(a) and 1(b) illustrate the emission spectra for the fluorescein (a) and rhodamine (b) components of compound 11 (Example 1) before and after β -lactamase cleavage of the β -lactam ring;

Fig. 2 illustrates the emission spectrum of compound 17 before and after β -lactamase cleavage of the β -lactam ring;

Fig. 3 illustrates the emission spectrum of compound 22 before and after β -lactamase cleavage of the β -lactam ring;

Fig. 4 illustrates the emission spectrum of compound 25 before and after β -lactamase cleavage of the β -lactam ring;

Fig. 5 illustrates the emission spectrum of compound CCF2 before and after β -lactamase cleavage of the β -lactam ring; and

Fig. 6 illustrates the emission spectrum of compound CCF1 before and after β -lactamase cleavage of the β -lactam ring.

Fig. 7A presents a table describing various nucleotide and amino acid sequences useful in the invention.

Fig. 7B-C depicts Sequence 1, the nucleotide and deduced amino acid sequence of *E. coli* RTEM as modified by Kadonaga et al (1984).

Fig. 7D-E depicts Sequence 2, the nucleotide and deduced amino acid sequence of Wild-type secreted RTEM enzyme with Ser2→Arg, Ala23→Gly.

Fig. 7F-G depicts Sequence 3, the nucleotide and deduced amino acid sequence of RTEM enzyme with β -globin upstream leader, mammalian Kozak sequence, replacement of signal sequence by Met Gly.

Fig. 7H-I depicts Sequence 4, the nucleotide and deduced amino acid sequence of RTEM β -lactamase with mammalian Kozak sequence and replacement of signal sequence by Met Asp.

Fig. 7J-K depicts Sequence 5, the nucleotide and deduced amino acid sequence of *Bacillus licheniformis* β -lactamase with signal sequence replaced.

DESCRIPTION OF THE INVENTION

DEFINITIONS

In accordance with the present invention and as used herein, the following terms are defined with the following meanings, unless stated otherwise.

The term "fluorescent donor moiety" refers the radical of a fluorogenic compound which can absorb energy and is capable of transferring the energy to another fluorogenic molecule or part of a compound. Suitable donor fluorogenic molecules include, but are not limited to, coumarins and related dyes xanthene dyes such as fluoresceins, rhodols, and rhodamines, resorufins, cyanine dyes, bimanes, acridines, isoindoles, dansyl dyes, aminophthalic hydrazides such as luminol and isoluminol derivatives, aminophthalimides, aminonaphthalimides, aminobenzofurans, aminoquinolines, dicyanohydroquinones, and europium and terbium complexes and related compounds.

The term "quencher" refers to a chromophoric molecule or part of a compound which is capable of reducing the emission from a fluorescent donor when attached to the donor. Quenching may occur by any of several mechanisms including fluorescence resonance energy transfer, photoinduced electron transfer, paramagnetic enhancement of intersystem crossing, Dexter exchange coupling, and exciton coupling such as the formation of dark complexes. The term "acceptor" as used herein refers to a quencher which operates via fluorescence resonance energy transfer. Many acceptors can re-

emit the transferred energy as fluorescence. Examples include coumarins and related fluorophores, xanthenes such as fluoresceins, rhodols, and rhodamines, resorufins, cyanines, difluoroboradiazaindacenes, and phthalocyanines. Other chemical classes of acceptors generally do not re-emit the transferred energy. Examples include indigos, benzoquinones, anthraquinones, azo compounds, nitro compounds, indoanilines, di- and triphenylmethanes.

The term "dye" refers to a molecule or part of a compound which absorbs specific frequencies of light, including but not limited to ultraviolet light. The terms "dye" and "chromophore" are synonymous.

The term "fluorophore" refers to chromophore which fluoresces.

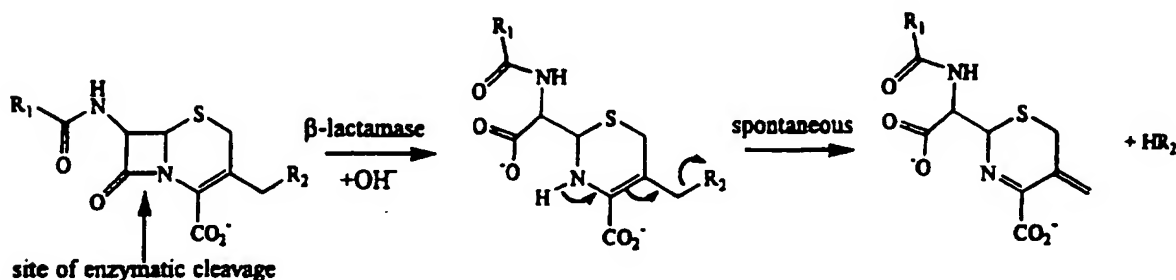
The term "membrane-permeant derivative" means a chemical derivative of a compound of general formula wherein at least one of X and Y contains at least one acylated aromatic hydroxyl, acylated amine, or alkylated aromatic hydroxyl wherein the acyl group contains 1 to 5 carbon atoms and wherein the alkyl group is selected from the group consisting of $-\text{CH}_2\text{OC}(\text{O})\text{alk}$, $-\text{CH}_2\text{SC}(\text{O})\text{alk}$, $-\text{CH}_2\text{OC}(\text{O})\text{Oalk}$, lower acyloxy-alpha-benzyl, and deltabutyrolactonyl; wherein alk is lower alkyl of 1 to 4 carbon atoms. These derivatives are made better able to cross cell membranes, i.e. membrane permeant, because hydrophilic groups are masked to provide more hydrophobic derivatives. Also, the masking groups are designed to be cleaved from the fluorogenic substrate within the cell to generate the derived substrate intracellularly. Because the substrate is more hydrophilic than the membrane permeant derivative it is now trapped within the cells.

The term "alkyl" refers to straight, branched, and cyclic aliphatic groups of 1 to 8 carbon atoms, preferably 1 to 6 carbon atoms, and most preferably 1 to 4 carbon atoms. The term "lower alkyl" refers to straight and branched chain alkyl groups of 1 to 4 carbon atoms.

The term "aliphatic" refers to saturated and unsaturated alkyl groups of 1 to 10 carbon atoms, preferably 1 to 6 carbon atoms, and most preferably 1 to 4 carbon atoms.

SUBSTRATES

β -Lactamases are nearly optimal enzymes in respect to their almost diffusion-controlled catalysis of β -lactam hydrolysis [Christensen, H. et al., *Biochem. J.* 266: 853-861 (1990)]. Upon examination of the other properties of this class of enzymes, it was determined that they were suited to the task of an intracellular reporter enzyme. They cleave the β -lactam ring of β -lactam antibiotics, such as penicillins and cephalosporins, generating new charged moieties in the process [O'Callaghan, C.H. et al., *Antimicrob. Agents. Chemother.* 8: 57-63, (1968); Stratton, C.W., *J. Antimicrob. Chemother.* 22, Suppl. A: 23-35 (1988)]. A first generation cephalosporin is illustrated below, left, with the arrow pointing to the site of cleavage by β -lactamase. The free amino group thus generated (middle structure below) donates electron density through the vinyl group to promote irreversible cleavage of a nucleofugal group R_2 from the 3'-position. R_2 is thus free to diffuse away from the R_1 -cephalosporin conjugate (right-hand structure below).



β -Lactamases are a class of enzymes that have been very well characterized because of their clinical relevance in making bacteria resistant to β -lactam antibiotics [Waley, S.G., *Sci. Prog.* 72: 579-597 (1988); Richmond, M.H. et al., *Ann. N.Y. Acad. Sci.* 182: 243-257 (1971)]. Most β -lactamases have been cloned and their amino acid sequence determined [see, e.g., Ambler, R.P., *Phil. Trans. R. Soc. Lond. [Ser.B.]* 289: 321-331 (1980)].

A gene encoding β -lactamase is known to molecular biologists as the ampicillin resistance gene (Amp^r) and is

commonly used to select for successfully transduced bacteria [Castagnoli, L. et al., *Genet.Res.* 40: 217-231 (1982)]; clones thereof are almost universally available. The enzyme catalyzes the hydrolysis of a β -lactam ring and will not accept peptides or protein substrates [Pratt, R.F. and Govardhan, C.P., *Proc. Natl. Acad. Sci.USA* 81: 1302-1306 (1984); Murphy, B.P. and Pratt, R.F., *Biochemistry* 30: 3640-3649 (1991)]. The kinetics of this reaction is well understood and there is no product inhibition [Bush, K. and Sykes, R.B., *Antimicrob. Agents. Chemother.* 30: 6-10 (1986); Christensen et al. (1990), *supra*]. The enzyme substrates are less polar than the products.

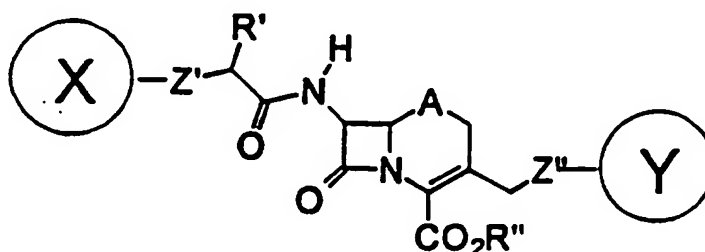
The carboxyl group in the substrate can be easily masked by an acetoxymethyl ester [Jansen, A.B.A. and Russell, T.J., *J.Chem.Soc.* 2127-2132, (1965); Daehne, W. et al., *J.Med.Chem.* 13: 607-612 (1970)], which is readily cleaved by endogenous mammalian intracellular esterases. Conversion by these esterases followed by the β -lactam cleavage by β -lactamase generates two negative charges and a tertiary amine, which protonates. To date, there has been no report of a fluorogenic substrate with the appropriate properties, but multiple chromogenic substrates of different design have been reported and are commercially available [Jones, R.N. et al., *J.Clin.Microbiol.* 15: 677-683 (1982); Jones, R.N. et al., *J.Clin.Microbiol.* 15: 954-958 (1982); O'Callaghan, C.H. et al., *Antimicrob.Agents.Chemother.* 1: 283-288 (1972)].

A large number of β -lactamases have been isolated and characterized, all of which would be suitable for use in accordance with the present invention. Initially, β -lactamases were divided into different classes (I through V) on the basis of their substrate and inhibitor profiles and their molecular weight [Richmond, M.H. and Sykes, R.B., *Adv.Microb.Physiol.* 9: 31-88 (1973)]. More recently, a classification system based on amino acid and nucleotide sequence has been introduced [Ambler, R.P., *Phil. Trans. R. Soc. Lond. [Ser.B.]* 289: 321-331 (1980)]. Class A β -lactamases possess a serine in the active site and have an approximate weight of 29kd. This class contains the plasmid-

mediated TEM β -lactamases such as the RTEM enzyme of pBR322. Class B β -lactamases have an active-site zinc bound to a cysteine residue. Class C enzymes have an active site serine and a molecular weight of approximately 39kd, but have no amino acid homology to the class A enzymes.

The coding region of an exemplary β -lactamase employed in the reporter gene assays described herein is indicated in SEQ ID NO:1 (nucleic acid sequence) and SEQ ID NO:2 (amino acid sequence). The pTG2del1 containing this sequence has been described [Kadonaga, J.T. et al., *J.Biol.Chem.* 259: 2149-2154 (1984)]. The entire coding sequence of wildtype pBR322 β -lactamase has also been published [Sutcliffe, J.G., *Proc.Natl.Acad.Sci.USA* 75: 3737-3741 (1978)]. As would be readily apparent to those skilled in the field, this and other comparable sequences for peptides having β -lactamase activity would be equally suitable for use in accordance with the present invention. The β -lactamase reporter gene is employed in an assay system in a manner well known per se for the use of reporter genes (for example, in the form of a suitable plasmid vector).

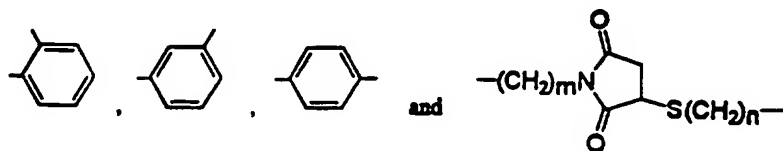
In conjunction with a suitable β -lactamase, there are employed in accordance with the present invention fluorogenic substrates of the general formula I



in which one of X and Y is a fluorescent donor moiety and the other is a quencher (which may or may not re-emit); R' is selected from the group consisting of H, lower alkyl, $(CH_2)_nOH$, $(CH_2)_nCOOR''$, and $=NOJ$, in which n is 0 or an integer from 1 to 5 and J is H, Me, CH_2COOH , $CHMeCOOH$, and CMe_2COOH ; R'' is selected from the group consisting of H, physiologically acceptable metal and ammonium cations, $-CHR^2OCO(CH_2)_nCH_3$, $-CHR^2OCOC(CH_3)_3$, acylthiomethyl, acyloxy-alpha-benzyl, delta-

butyrolactonyl, methoxycarbonyloxymethyl, phenyl, methylsulphinylmethyl, beta-morpholinoethyl, dialkylaminoethyl, and dialkylaminocarbonyloxymethyl, in which R^2 is selected from the group consisting of H and lower alkyl; A is selected from the group consisting of S, O, SO, SO₂ and CH₂; and Z' and Z'' are linkers for the fluorescent donor and quencher moieties.

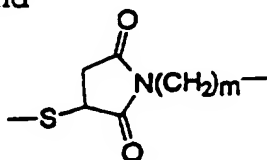
The linkers Z' and Z'' serve the purpose of attaching the fluorescent donor and quencher moieties to the cephalosporin-derived backbone, and may facilitate the synthesis of the compounds of general formula I. In general formula I, Z' may represent a direct bond to the backbone; alternatively, suitable linkers for use as Z' include, but are not limited to, the following: $-(CH_2)_nCONR^2(CH_2)_m-$, $-(CH_2)_nNR^2CO(CH_2)_m-$, $-(CH_2)_nNR^3CONR^2(CH_2)_m-$, $-(CH_2)_nNR^3CSNR^2(CH_2)_m-$, $-(CH_2)_nCONR^3(CH_2)_pCONR^2(CH_2)_m-$, $-(CH_2)_n-$, $-(CH_2)_nNR^3CO(CH_2)_pS(CH_2)_m-$, $-(CH_2)_nS(CH_2)_m-$, $-(CH_2)_nO(CH_2)_m-$, $-(CH_2)_nNR^2(CH_2)_m-$, $-(CH_2)_nSO_2NR^2(CH_2)_m-$, $-(CH_2)_nCO_2(CH_2)_m-$,



wherein R^2 and n are as previously defined; R^3 is selected from the group consisting of hydrogen and lower alkyl; and each of m and p is independently selected from the group consisting of 0 and integers from 1 to 4. Especially preferred are Z' groups such where n and m are 0. Also particularly preferred are such Z' groups where R^2 is H.

Suitable linkers Z'' for the Y moiety include, but are not limited to, a direct bond to a heteroatom (e.g., O, N or S) in the dye's chromophore or the following:

$-O(CH_2)_n-$, $-S(CH_2)_n-$, $-NR^2(CH_2)_n-$, $-N^+R^2_2(CH_2)_n-$, $-OCONR^2(CH_2)_n-$, $-O_2C(CH_2)_n-$, $-SCSNR^2(CH_2)_n-$, $-SCSO(CH_2)_n-$, $-S(CH_2)_nCONR^2(CH_2)_m$, $-S(CH_2)_nNR^2CO(CH_2)_m$, and



in which R^2 , n and m are as previously defined; and m is an integer from 0 to 4. Particularly preferred Z^n groups are $-S(CH_2)_n$. Especially preferred is H.

Preferred R' groups include H and methyl.

- 5 Particularly preferred is H. Preferred R'' groups include H and acetoxymethyl. A preferred R^2 group is H. A preferred A group is $-S-$.

In a preferred aspect, the compounds of the present invention are membrane-permeant. Particularly preferred are
10 such compounds wherein at least one of X and Y contains at least one acylated aromatic hydroxyl, acylated amine, or alkylated aromatic hydroxyl wherein the acyl group contains 1 to 5 carbon atoms and wherein the alkyl group is selected from the group consisting of $-CH_2OC(O)alk$, $-CH_2SC(O)alk$, $-CH_2OC(O)Oalk$, lower acyloxy- α -benzyl, and δ -butyrolactonyl, wherein alk is lower alkyl of 1 to 4 carbon
15 atoms. Particularly preferred are such compounds where at least one of X and Y contains at least one acylated aromatic hydroxy, wherein the acyl group is either acetyl, n -propionyl, or n -butyryl. Also particularly preferred are such compounds
20 wherein at least one of X and Y contains an acetoxy methyl group on an aromatic hydroxyl group.

In another preferred aspect, the quencher or acceptor is a fluorescein, rhodol, or rhodamin of formulae
25 VIII-XII. Preferred are such compounds where the donor is a fluorescein of formula VIII and the quencher or acceptor is a rhodol or rhodamine of formulae VIII-XII. Also preferred are such compounds where the donor is a fluorescein of formula VIII and the quencher or acceptor is a tetrahalo fluorescein
30 of formula VIII in which R^a , R^b , R^c , and R^d are independently Br or Cl. Also preferred are such compounds where the quencher or acceptor is a rhodol of formulae VIII, IX, and XI. Another preferred group of such compounds are those where the quencher or acceptor is a rhodamine of formulae VIII, X, and
35 XII.

In a another preferred aspect, the donor is a coumarin of formulae II-VII and the quencher/acceptor is a fluorescein, rhodol, or rhodamine of formulae VIII-XII, XLVII,

or XLVII, and membrane-permeant fluorogenic derivatives thereof. Particularly preferred are such compounds with a fluorescein quencher/acceptor of formula VIII. Especially preferred are such compounds where the coumarin is 7-hydroxycoumarin or 7-hydroxy-6-chlorocoumarin and the fluorescein acceptor is fluorescein or dichlorofluorescein.

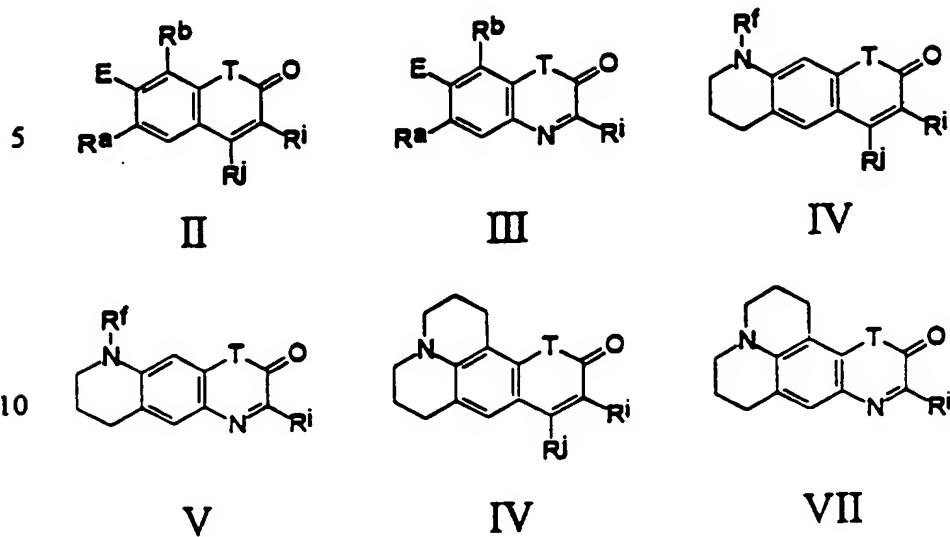
As would readily be appreciated by those skilled in the art, the efficiency of fluorescence resonance energy transfer depends on the fluorescence quantum yield of the donor fluorophore, the donor-acceptor distance and the overlap integral of donor fluorescence emission and acceptor absorption. The energy transfer is most efficient when a donor fluorophore with high fluorescence quantum yield (preferably, one approaching 100%) is paired with an acceptor with a large extinction coefficient at wavelengths coinciding with the emission of the donor. The dependence of fluorescence energy transfer on the above parameters has been reported [Forster, T. (1948) *Ann. Physik* 2: 55-75; Lakowicz, J.R., *Principles of Fluorescence Spectroscopy*, New York: Plenum Press (1983); Herman, B., *Resonance energy transfer microscopy*, in: *Fluorescence Microscopy of Living Cells in Culture, Part B, Methods in Cell Biology*, Vol 30, ed. Taylor, D.L. & Wang, Y.-L., San Diego: Academic Press (1989), pp. 219-243; Turro, N.J., *Modern Molecular Photochemistry*, Menlo Park: Benjamin/Cummings Publishing Co., Inc. (1978), pp. 296-361], and tables of spectral overlap integrals are readily available to those working in the field [for example, Berlman, I.B. *Energy transfer parameters of aromatic compounds*, Academic Press, New York and London (1973)]. The distance between donor fluorophore and acceptor dye at which fluorescence resonance energy transfer (FRET) occurs with 50% efficiency is termed R_0 and can be calculated from the spectral overlap integrals. For the donor-acceptor pair fluorescein - tetramethyl rhodamine which is frequently used for distance measurement in proteins, this distance R_0 is around 50-70 Å [dos Remedios, C.G. et al. (1987) *J. Muscle Research and Cell Motility* 8:97-117]. The distance at which the energy transfer in this pair exceeds 90% is about 45 Å. When attached to the

cephalosporin backbone the distances between donors and acceptors are in the range of 10 Å to 20 Å, depending on the linkers used and the size of the chromophores. For a distance of 20 Å, a chromophore pair will have to have a calculated R_0 of larger than 30 Å for 90% of the donors to transfer their energy to the acceptor, resulting in better than 90% quenching of the donor fluorescence. Cleavage of such a cephalosporin by β -lactamase relieves quenching and produces an increase in donor fluorescence efficiency in excess of tenfold.

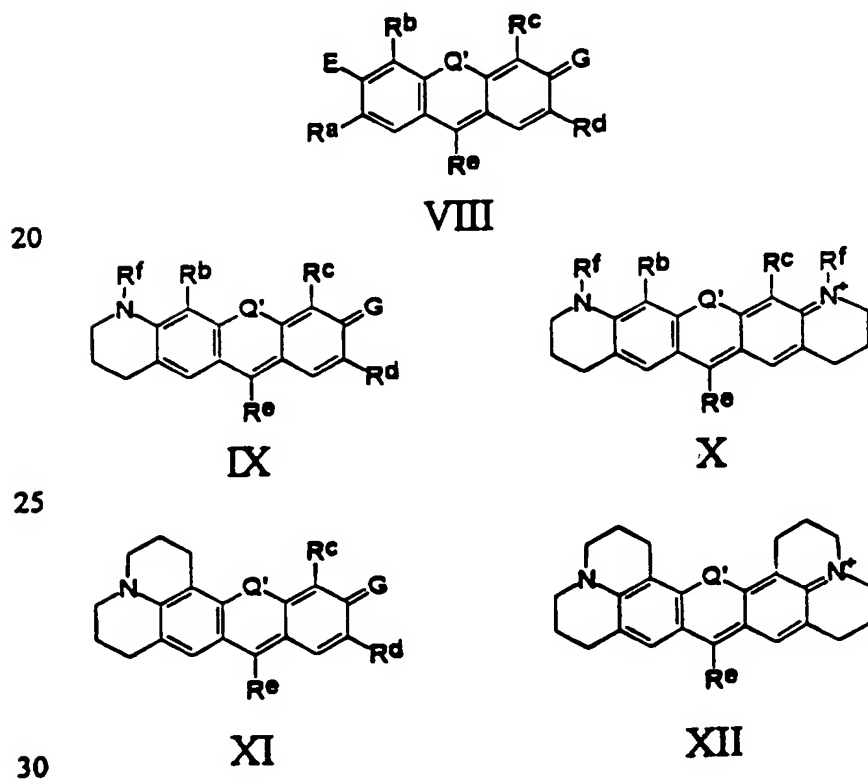
Accordingly, it is apparent that identification of appropriate donor-acceptor pairs for use as taught herein in accordance with the present invention would be essentially routine to one skilled in the art.

To measure β -lactamase activity in the cytoplasm of living cells, smaller molecular weight chromophores as hereinafter described are in general preferred over larger ones as substrate delivery becomes a problem for larger compounds. Large molecules, especially those over about 1200 daltons, also tend to bind more avidly to cellular constituents than small ones, thereby removing at least some of them from access and cleavage by β -lactamase.

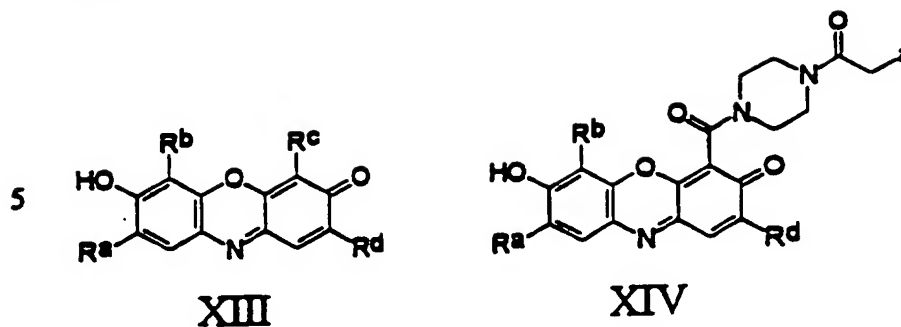
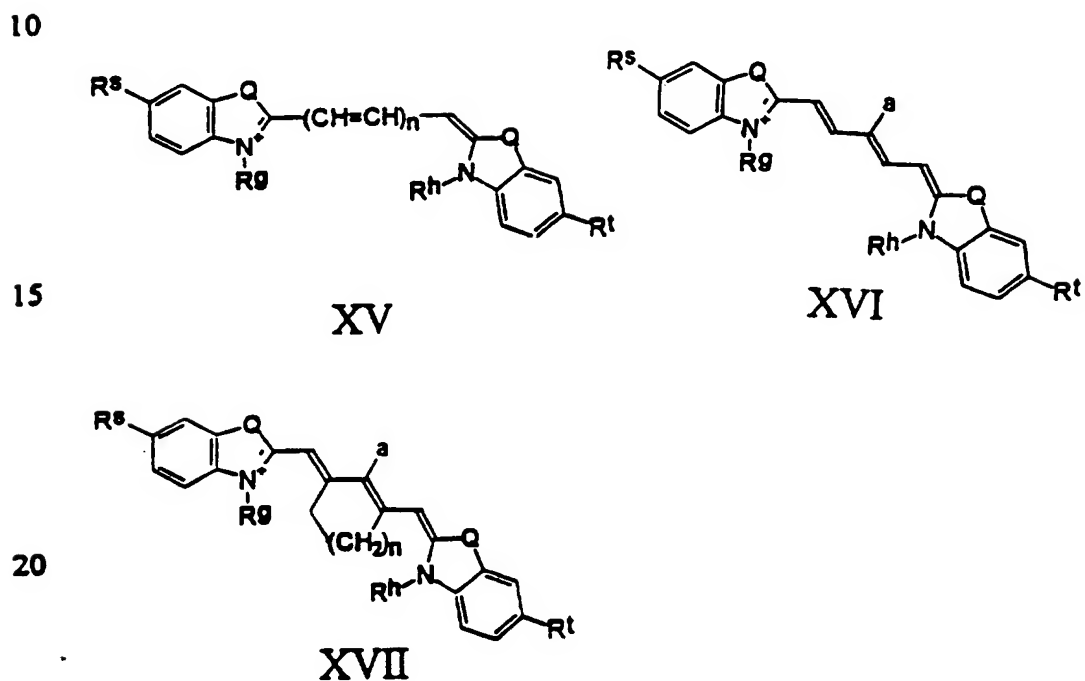
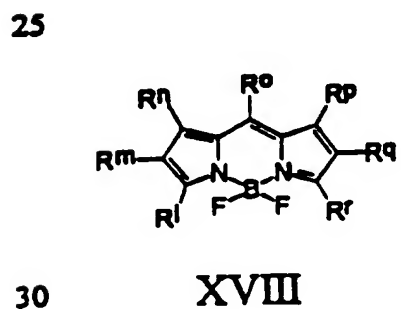
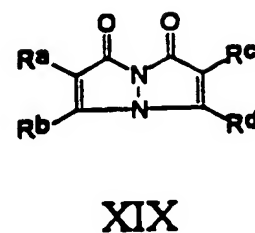
Chromophores suitable for use as X and Y are well known to those skilled in the art. Generic structures of particular classes of chromophores suitable for use as X and Y are provided below. Compounds of general formulas II - XXXIV are exemplary of fluorophores which serve as the basis for particularly suitable donor moieties in the compounds of general formula I. Suitable chromophores for use as the basis of acceptor moieties in the compounds of general formula include, but are not limited to, compounds of general formulas II - LIV. Chromophores of general formulae XXXV-LIV usually do not re-emit efficiently.

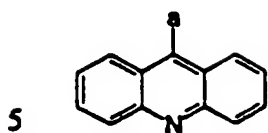
Coumarins and related dyesXanthene dyes (including fluoresceins, rhodols and rhodamines)

15

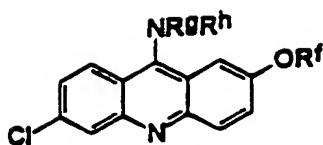


18

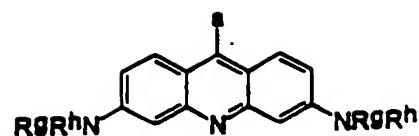
ResorufinsCyanine dyesDifluoroboradiazaindacene dyesBimanes

Acridines

XX



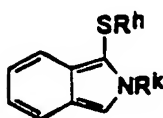
XXI



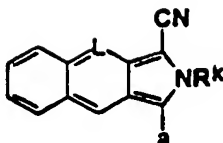
XXII

Isoindoles

10



XXIII

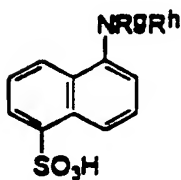


XXIV

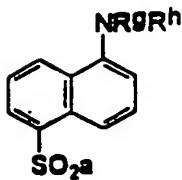
15

Dansyl dyes

20



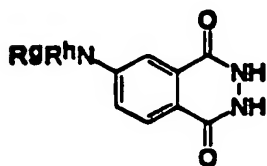
XXV



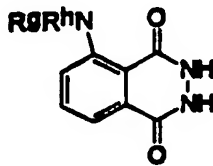
XXVI

Aminophthalic hydrazides (luminol and isoluminol derivatives)

25

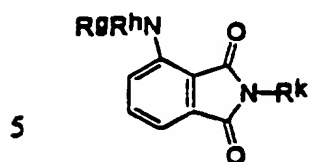


XXVII

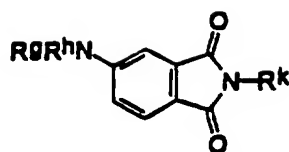


XXVIII

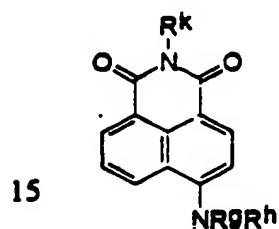
30

Aminophthalimides

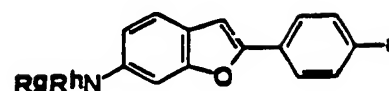
XXIX



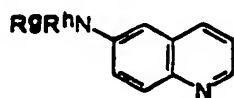
XXX

10 Aminonaphthalimides

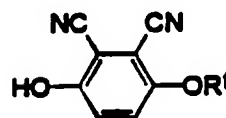
XXXI

Aminobenzofurans

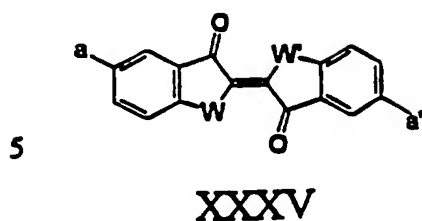
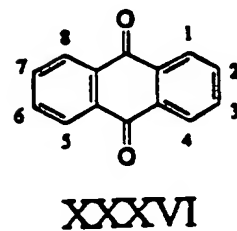
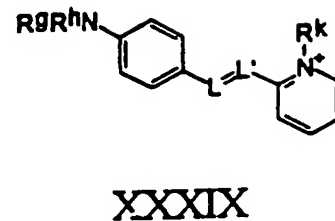
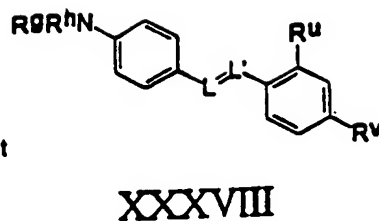
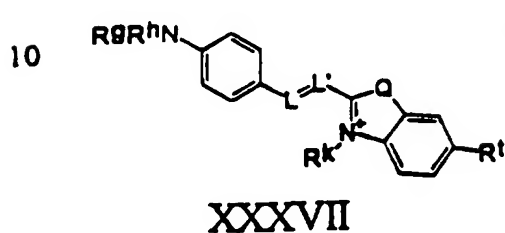
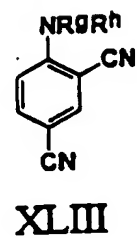
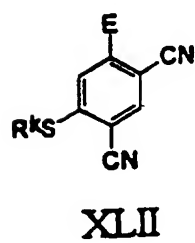
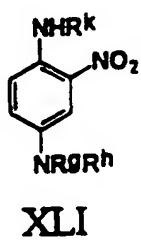
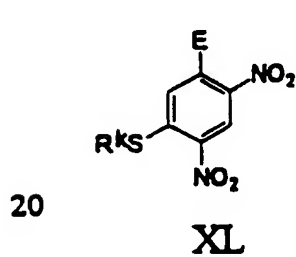
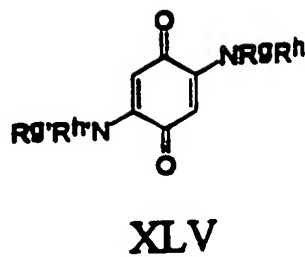
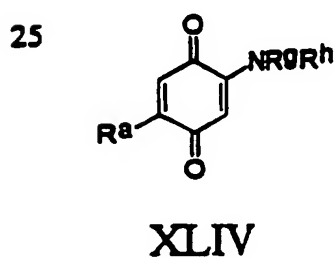
XXXII

20 Aminoquinolines

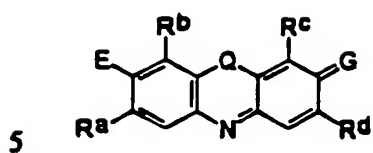
XXXIII

Dicyanohydroquinones

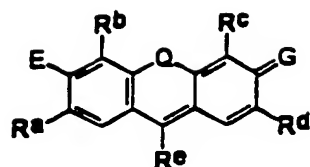
XXXIV

Indigo dyesAnthraquinone dyesPolymethine dyes15 Nitro dyes and cyano derivativesQuinone dyes

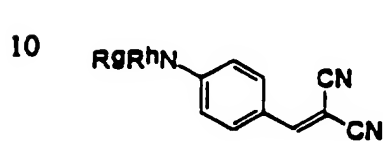
30

Xanthene dyes

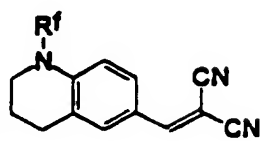
XLVI



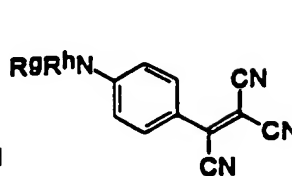
XLII

Dicyanovinyl and tricyanovinyl dyes

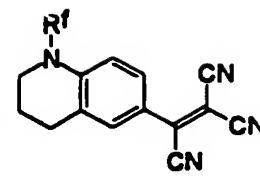
XLVIII



XLIX

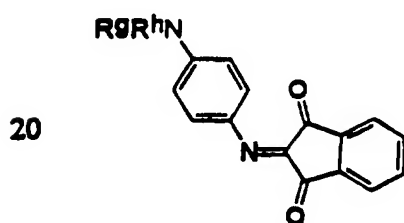


L

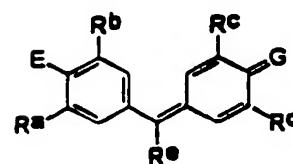


LI

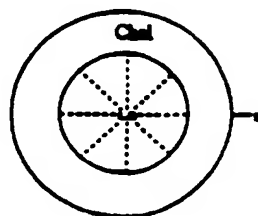
15

Indoaniline dyes (ninhydrin derivatives)Di- and triphenylmethane dyes

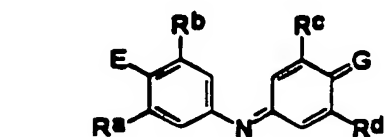
LII



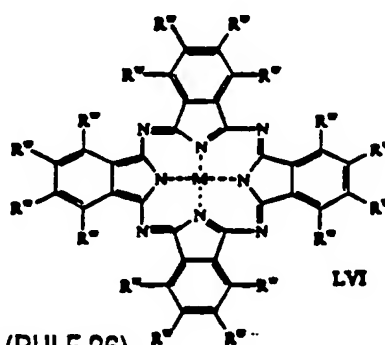
LIII



LV

Indamines and related dyes

LIV



LVI

In preferred embodiments of the compounds of general formulas II - LVI:

each of a and a' is independently H or an attachment point (i.e., a location at which the dye moiety is attached to the core structure of general formula I;

E is selected from the group consisting of H, OH, OR^k and NR^gR^h;

G is selected from the group consisting of O and N⁺R^g'R^h';

each of L and L' is independently selected from the group consisting of CH and N;

M is selected from the group consisting of H, Mg, Al, Si, Zn, and Cu;

Q is selected from the group consisting of O, S, C(CH₃)₂ and NR^g;

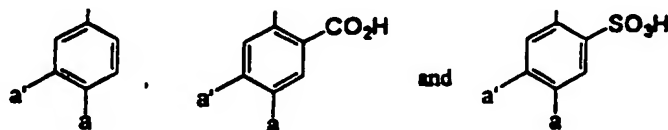
Q' is selected from the group consisting of O, CH₂, C(CH₃)₂, NR^k and SO₂;

T is selected from the group consisting of O and NR^k;

each of W and W' is selected from the group consisting of O, S, Se and NH;

each of R^a, R^b, R^c and R^d is independently selected from the group consisting of an attachment point, H, halogen and lower alkyl;

R^e is selected from the group consisting of an attachment point, H, lower alkyl, (CH₂)_nCO₂H, (CH₂)_nCHaCO₂H, CHa(CH₂)_nCO₂H, (CH₂)_nCOa, CH=CHCOa,

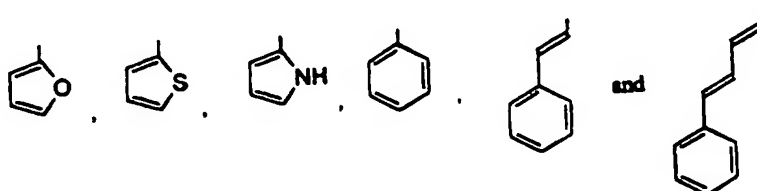


each of R^f, R^g, R^g', R^h, R^h' and R^k is independently selected from the group consisting of an attachment point, H, lower alkyl and CH₂(CH₂)_na;

Rⁱ is selected from the group consisting of an attachment point, H, halogen, lower alkyl, CN, CF₃, phenyl, CO₂H and CONR^g'R^h';

R^j is selected from the group consisting of an attachment point, H, halogen, lower alkyl, CN, CF_3 , phenyl, CH_2CO_2H , $CH_2CONR^{g'}R^{h'}$;

5 each of R^i and R^f is independently selected from the group consisting of an attachment point, H, lower alkyl,



10

each of R^m , R^n , R^p and R^q is independently selected from the group consisting of an attachment point, H, lower alkyl and phenyl;

15 R^o is selected from the group consisting of an attachment point, H and lower alkyl;

each of R^s and R^t is independently selected from the group consisting of an attachment point, H, halogen, lower alkyl and OR^f ;

20 each of R^u and R^v is independently selected from the group consisting of an attachment point, H, halogen, CN and NO_2 ;

each of R^w is independently selected from the group consisting of an attachment point, H, COO^- , SO_3^- , and PO_3^{2-} .

25 Ln is selected from the group consisting of Eu^{3+} , Ln^{3+} , and Sm^{3+} ;

30 Chel is a polydentate chelator with at least six and preferably eight to ten donor atoms that can face into a cavity of diameter between 4 and 6 angstroms, which may or may not be macrocyclic, which includes a chromophore absorbing between 300 and 400 nm, and which includes an attachment point through which Chel can be conjugated to Z' or Z'' . A suitable Chel moiety is a europium tris-(bipyridine) cryptands. In the anthraquinone chromophores of general formula XXXIX, each of positions 1-8 may carry a substituent H or E, or serve as an attachment point.

35

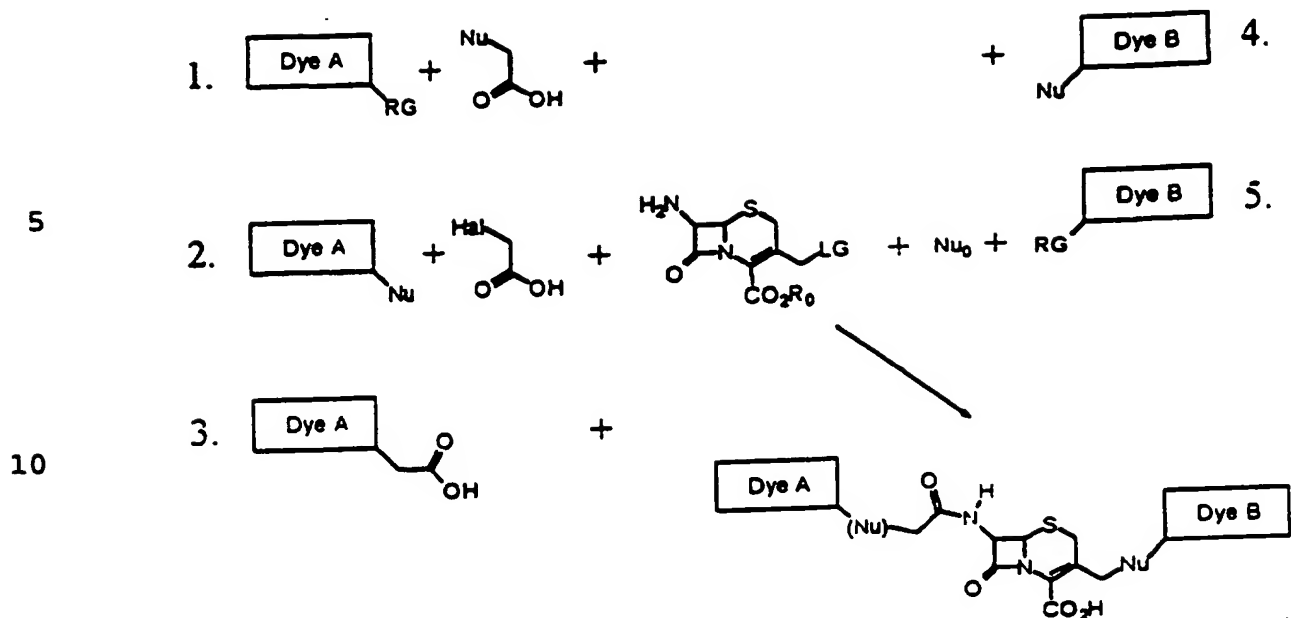
Europium tris-(bipyridine) cryptand donors may be suitably paired with acceptors of the formulae XV-XVII, XXXVI, XLVI-XLVII, LIV, and LVI. Terbium tris-(bipyridine) cryptand

donors may be suitably paired with acceptors of the formulae VIII-XVIII, XXXVI-XLI, and XLV-LIV, and LVI.

The Europium tris-(bipyridine) cryptand/
phtalocyanines donor/acceptor pair may be of particular
5 interest when it is desirable to measure β -lactamase activity
by emission of energy in the near to far red range.

In many applications it is desirable to derivatize
compounds of general formula I to render them hydrophobic and
permeable through cell membranes. The derivatizing groups
10 should undergo hydrolysis inside cells to regenerate the
compounds of general formula I and trap them inside the cells.
For this purpose, it is preferred that any phenolic hydroxyls
or free amines in the dye structures are acylated with C₁-C₄
acyl groups (e.g. formyl, acetyl, n-butyryl) or converted to
15 various other esters and carbonates [for examples, as
described in Bundgaard, H., Design of Prodrugs, Elsevier
Science Publishers (1985), Chapter I, page 3 et seq.].
Phenols can also be alkylated with 1-(acyloxy)alkyl,
acylthiomethyl, acyloxy-alpha-benzyl, deltabutyrolactonyl, or
20 methoxycarbonyloxymethyl groups. In the case of fluoresceins,
rhodols, and rhodamines this manipulation is particularly
useful, as it also results in conversion of the acid moiety in
these dyes to the spirolactone. To promote membrane
permeation, the carboxyl at the 4-position of the
25 cephalosporin should be esterified with 1-(acyloxy)alkyl,
acylthiomethyl, acyloxy-alpha-benzyl, delta-butyrolactonyl,
methoxycarbonyloxymethyl, phenyl, methylsulfinylmethyl, beta-
morpholionethyl, 2-(dimethylamino)ethyl, 2-
(diethylamino)ethyl, or dialkylaminocarbonyloxymethyl groups
30 as discussed in Ferres, H. (1980) *Chem. Ind.* 1980: 435-440.
The most preferred esterifying group for the carboxyl is
acetoxymethyl.

A general method for synthesis of compounds of
general formula I is depicted below. As one of ordinary skill
35 in the art will appreciate, the methods below can be used for
a variety of derivatives, and other methods of synthesis are
possible.



In these compounds, RG is a nucleophile-reactive group (e.g., iodoacetamide, isocyanate, isothiocyanate, etc.); Nu is a nucleophile (e.g., -SH, -NH₂, -OH, etc.); R₀ is H or an ester group (e.g., benzhydryl ester, tertiary butyl ester, etc.); Nu₀ is a bidentate nucleophile (e.g., HS⁻, HSCH₂CH₂NH₂, xanthate, etc.); and Hal is a halogen (e.g., chlorine, bromine or iodine).

The cephalosporin starting materials are commercially available cephalosporin derivatives 7-aminocephalosporanic acid or 7-amino 3'-chlorocephalosporanic acid as its benzhydryl or tertiary butyl ester (R_0). Prior to coupling the dyes A and B carrying nucleophile reactive groups (RG) it is sometimes advantageous to esterify or alkylate their phenolic and free amine residues. The order of attaching dye A and dye B depends on the choice of reagents. Dye A is tethered to the cephalosporin via an alkyl amide linker. This is achieved by reacting a dye A carrying a nucleophile-reactive group (RG) with a bifunctional aliphatic acid (e.g., amino-, mercapto- or hydroxyalkyl acid) and coupling of the acid to the cephalosporin 7-amine (path 1). Alternatively, dye A carrying a nucleophilic group (e.g.,

amine or thiol) is reacted with a halogenated alkyl acid and the acid coupled to the cephalosporin 7-amine (path 2). In both pathways, the order of the two reactions can be reversed. Dyes A containing an aliphatic acid can be directly coupled to the cephalosporin (path 3). Dye B carrying a nucleophilic substituent can be coupled to the 3'-position in the cephalosporin by direct displacement of the leaving group (LG) (path 4). A Dye B carrying a nucleophile-reactive group can be reacted with a bidentate nucleophile which is coupled then attached to the cephalosporin by leaving group (LG) displacement (path 5); the order of the reactions can be reversed.

In some cases it might be necessary to conduct the first reaction with a bidentate nucleophile with one of its nucleophilic groups masked. The second coupling is then performed after removal of that protection group. After attachment of both dyes the cephalosporin ester is cleaved (in cases where R_0 is not H). To make membrane permeant substrates the acid is then re-esterified to esters that can be deprotected by the cytoplasmic environment of a mammalian cell. For applications not involving cell cytoplasm, any remaining acyl and alkyl groups that were used to mask phenols and free amines on the dyes are removed.

Preferred combinations of classes of donors and acceptors suitable for use in accordance with the present invention are indicated in Table 1. In embodiments of compounds of general formula I using these combinations, fluorescent resonant energy transfer (FRET) occurs. Of course, as would be readily understood by those working in the field, many other combinations of donors and acceptors/quenchers (including those that re-emit and those that do not) would be suitable for use in accordance with the present invention. In general, suitable donor and acceptor pairs are those where the donor's emission spectrum significantly overlaps the acceptor's excitation spectrum.

TABLE 1

DONORS

5	ACCEPTORS	II - VIII, XIX - XXI, XXIII - XXXIV	VII - XIV, XVII, XXII	XV - XVI, LV
		II - VIII, XIX - XXI, XXIII - XXXIV	FRET	
10		VII - XIV, XVII, XXII	FRET	FRET
		XV - XVII	FRET	FRET
		XL - XLV, XLVII - LII	FRET	FRET
15		XXXV-XXXIX, XLVI-XLVII, LIII-LIV, LVI	FRET	FRET
				FRET

Fluorescent donor moieties of particular interest include coumarins and fluoresceins. Particular quenchers of interest include fluoresceins, rhodols and rhodamines. Combinations of interest include the use of a coumarin donor with a fluorescein, rhodol or rhodamine quencher, and a fluorescein donor with a rhodol or rhodamine quencher. Specific combinations of interest include the following: a coumarin (e.g., 7-hydroxycoumarin) or chloro derivative thereof with a fluorescein or dichloro derivative thereof; a fluorescein with an eosin or tetrachlorofluorescein; a fluorescein with a rhodol derivative; and a rhodamine with a fluorescein.

Europium chelate donors may be suitably paired with acceptors of the formulae XV-XVII, XXXVI, XLVI-XLVII, LIV, and LVI. Terbium chelate donors may be suitably paired with acceptors of the formulae VIII-XVIII, XXXVI-XLI, and XLV-LIV, and LVI. The europium and terbium chelate donors may be of particular interest for their very narrow emission peaks and their microsecond-to-millisecond excited state lifetimes, which can be readily discriminated from background fluorescence and scattering with excited-state lifetimes of nanoseconds or less.

In many applications it is desirable to derivatize compounds of general formula I to render them more hydrophobic and permeable through cell membranes. The derivatizing groups should undergo hydrolysis inside cells to regenerate the compounds of general formula I and trap them inside the cells. For this purpose, it is preferred that any phenolic hydroxyls or free amines in the dye structures are acylated with C₁-C₄ acyl groups (e.g. formyl, acetyl, n-butyryl) or converted to various other esters and carbonates [for example, as described in Bundgaard, H., *Design of Prodrugs*, Elsevier Science Publishers (1985), Chapter 1, page 3 et seq.]. Phenols can also be alkylated with 1-(acyloxy)alkyl, acylthiomethyl, acyloxy-alpha-benzyl, delta-butyrolactonyl, or methoxycarbonyloxymethyl groups. In the case of fluoresceins, rhodols and rhodamines, acylation or alkylation of the free phenolic groups is particularly useful, as it also results in conversion of the acid moiety in these dyes to the spiro lactone. To promote membrane permeation, the carboxyl at the 4-position of the cephalosporin should be esterified with 1-(acyloxy)alkyl, acylthiomethyl, acyloxy-alpha-benzyl, delta-butyrolactonyl, methoxycarbonyloxymethyl, phenyl, methylsulfinylmethyl, beta-morpholinoethyl, 2-(dimethylamino)ethyl, 2-(diethylamino)ethyl, or dialkylaminocarbonyloxymethyl groups as discussed in Ferres, H. (1980) *Chem. Ind.* 1980: 435-440. The most preferred esterifying group for the carboxyl is acetoxymethyl.

The cephalosporin backbone serves as a cleavable linker between two dyes. After cleavage it provides the charges necessary to keep one of the two dyes inside the cell. Dyes are chosen in a manner that one dye absorbs light (quencher or acceptor chromophore) at the wavelength that the other one emits (donor fluorophore). In the intact cephalosporin the two dyes are in close proximity to each other. When exciting the donor fluorophore one observes fluorescence resonance energy transfer (FRET) from the donor to the acceptor instead of donor fluorescence [Forster, T., *Ann. Physik* 2: 55-75 (1948)]. If the acceptor is a nonfluorescent dye the energy is given off to the solvent; the

donor fluorescence is quenched. In the case of the acceptor being itself a fluorescent dye, fluorescence re-emission occurs at the acceptor's emission wavelength. In polar solvents such as water, hydrophobic donor and acceptor fluorophores can stack when separated by a short flexible linker. Due to this association in the ground state, a "dark complex" is formed [Yaron, A. et al., *Anal. Biochem.* 95: 228-235 (1979)]. In this complex, neither fluorophore can emit light, causing the fluorescence of both dyes to be quenched [Bojarski, C. and Sienicki, K. Energy transfer and migration in fluorescent solutions. In: *Photochemistry and Photophysics*, edited by Rabek, J.F. Boca Raton: CRC Press, Inc., 1990, pp. 1-57]. In either case, a large change in fluorescence goes along with β -lactam cleavage, which can be used to measure β -lactamase activity. As both dyes diffuse away from each other, stacking and energy transfer are disrupted. Cephalosporins carrying a donor and an acceptor dye which fluoresces are referred to herein as FRET-cephalosporins.

Fluorescence resonance energy transfer has been used as a spectroscopic ruler for measuring molecular distances in proteins and peptides as it is effective in the range from 10-100Å. This energy transfer is proportional to the inverse sixth power of the distance between donor and acceptor. Its efficiency is higher, the better donor emission and acceptor absorbance overlap, and the longer the fluorescence lifetime of the donor (in absence of the acceptor). FRET can be very efficient over distances of 10-20Å.

In the cephalosporin, distances for attachment of donor and acceptor are greater than 10Å and a minimum of 10 bond-lengths, if one includes the two minimal spacers at 7- and 3-positions. Over this distance FRET is very efficient, if the right donor-acceptor pairs are chosen. Conveniently, in a FRET-cephalosporin the 7-amine tethered dye stays attached to the polar hydrolysis products of cephalosporin cleavage, trapping it in the cells' cytoplasm. This position is best occupied by the donor fluorophore, although in some instances the acceptor may occupy this position. Upon

cleavage, fluorescence increases due to loss of the quencher dye.

The acceptor fluorophore is generally attached by a linker which imparts the greatest stability of the substrate to nucleophilic attack. A preferred linker is a thioether bond (-S-), which is very stable and due to its inductive effect reduces the reactivity of the β -lactam ring toward nucleophiles [Page, M.I., *Adv.Phys.Org.Chem.* 23: 165-270 (1987)]. In addition, the free thiol or thiolate group released upon hydrolysis often quenches the attached fluorophore, adding to the desired large change in fluorescence upon hydrolysis.

The fluorogenic substrates of the invention are initially colorless and nonfluorescent outside cells. The substrates are designed so they readily cross cell membranes into the cytoplasm, where they are converted to fluorescent compounds by endogenous nonspecific esterases and stay trapped due to their charges. In the intact molecules, fluorescence energy transfer occurs leading to fluorescence at a particular wavelength when the substrates are excited. Lactamase cleavage of the β -lactam ring is followed by expulsion of the fluorescein moiety with loss of fluorescence energy transfer. Excitation of the modified substrate now results in fluorescence at a different wavelength.

The assay systems of the present invention further provide an advantageous and rapid method of isolation and clonal selection of stably transfected cell lines containing reporter genes and having the desired properties which the transfection was intended to confer, e.g. fluorescent signal response after activation of a transfected receptor with a high signal-to-noise ratio from a high proportion of isolated cells. Current procedures for clonal selection of satisfactorily transfected, genetically engineered cells from the initial population, are done mainly by replica plating of colonies, testing of one set of colonies, visual selection of preferred clones, manual isolation of the replicas of the preferred clones by pipetting, and prolonged cellular cultivations. This procedure is laborious and time-consuming;

it may require several months to generate a clone useful for assays suited to drug screening. Moreover, it is difficult to manually select and maintain more than a few hundred clones. Using the assays of this present invention, the desired signal
5 from cellular beta-lactamase reporter system can be maintained within living and viable cells. Replica plating of colonies is unnecessary because single cells can be assayed and remain viable for further multiplication. Thus, from the population of initially transfected cells, one can rapidly select those
10 few individual living cells with the best fluorescent signal, using automated instruments such as a fluorescent-activated cell sorter, e.g. the Becton Dickinson FACS Vantage™. The selected cells are then collected for cultivation and propagation to produce a clonal cell line with the desired
15 properties for assays and drug screening.

As would be immediately apparent to those working in the field, the combination of a novel substrate in accordance with the invention and a suitable β -lactamase may be employed in a wide variety of different assay systems (such
20 as are described in U.S. Patent 4,740,459). In particular, the fluorogenic substrates of the invention enable the detection of β -lactamase activity in a wide variety of biologically important environments, such as human blood serum, the cytoplasm of cells and intracellular compartments;
25 this facilitates the measurement of periplasmic or secreted β -lactamase.

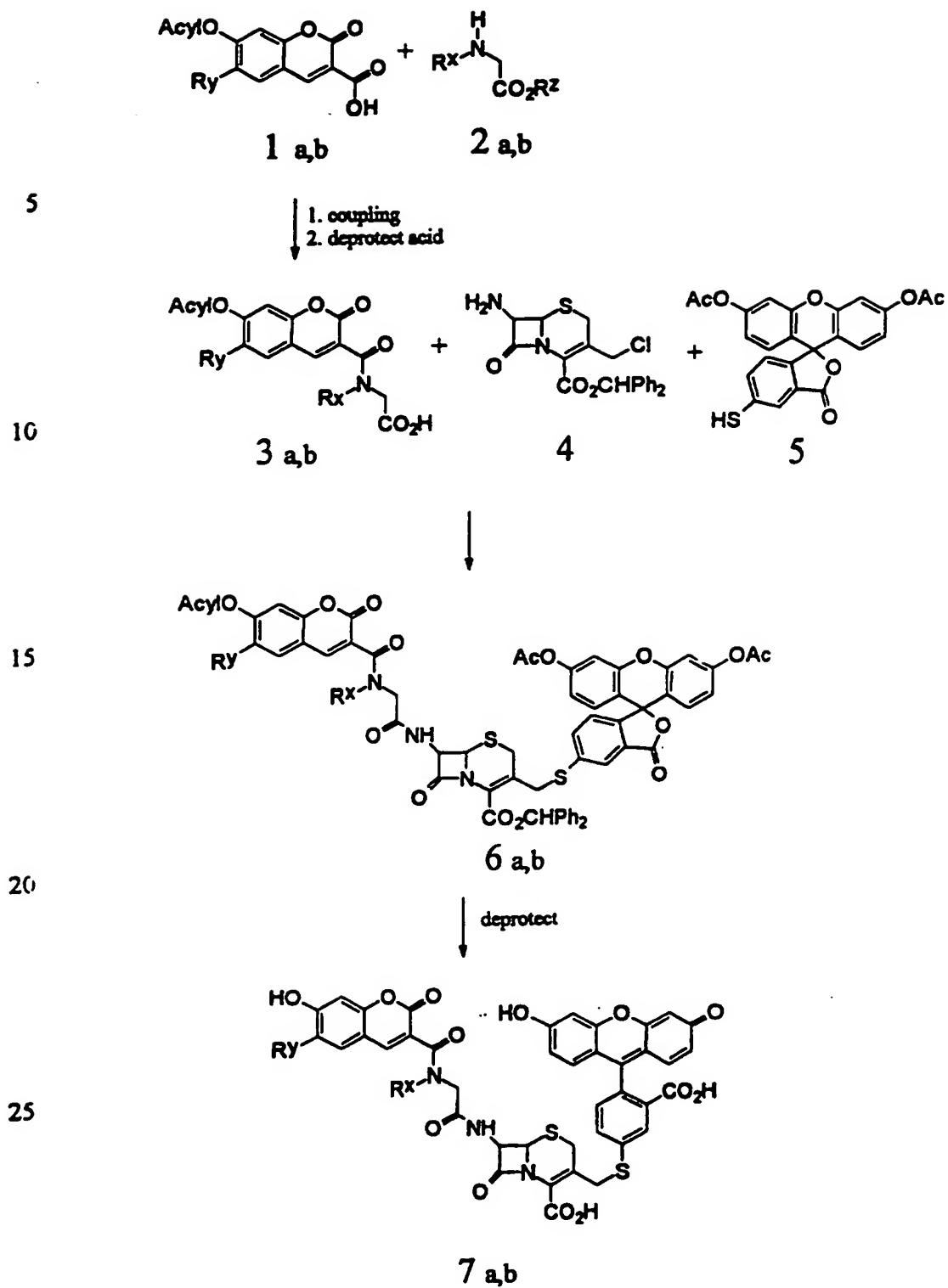
Further, the expression of any target protein can be detected by fusing a gene encoding the target protein to a β -lactamase gene, which can be localized by immunostaining and
30 fluorescence or electron microscopy. For example, β -lactamase fusion proteins may be detected in the lumen of organelles through the use of the substrates of the invention; only subcellular compartments containing the fusion protein fluoresce at a wavelength characteristic of the cleaved
35 substrate, whereas all others fluoresce at a wavelength characteristic of the intact molecule.

Both the intact and cleaved substrate are well retained in cells without the use of special measures, such as

chilling. The color change (even in individual small mammalian cells) is visible through a fluorescence microscope using normal color vision or photographic film; the fluorescence signal may be quantified and further enhanced by conventional digital image processing techniques. Moreover, because gene activation is detected not by a change in a single intensity but rather by a color change or a change in the ratio between two intensities at different wavelengths, the assays of the present invention are relatively immune to many artifacts such as variable leakiness of cells, quantity of substrate, illumination intensity, absolute sensitivity of detection and bleaching of the dyes.

A variety of substrates (e.g., compounds of general formulas 17, 22 and 25) have been prepared and their emission spectra obtained before and after β -lactamase cleavage. These substrates allow for β -lactamase detection primarily *in vitro*, as they bind strongly to serum and cellular proteins. Due to their hydrophobic nature, the fluorophores stack; this leads to a loss of fluorescence in the intact substrate. β -lactamase cleaves the substrates and relieves the stacking, allowing for fluorescence. Compounds (e.g., compound 11, Example 1) with reversed location of donor and acceptor fluorophore on the cephalosporin exhibit similar fluorescence behavior.

In one preferred embodiment of the invention, a compound of general formula 1 was coupled to a compound of general formula 2 to form a compound of general formula 3. Commercially-available compound 4 was then coupled to compound 3 using dicyclohexylcarbodiimide and the product reacted with compound 5, yielding a compound of general formula 6. Deprotection of compound 6 generated a compound of general formula 7. In exemplary embodiments, Acyl was acetyl, R^x was Me and R^y H (a), or Acyl was butyryl, R^x was H and R^y Cl (b); R^z was trimethylsilyl or benzyl.



The compounds of general formula 6 were modified to obtain membrane permeant derivatives which were converted to the corresponding fluorescent compounds of general formula 7 in intact cells due to the action of endogenous nonspecific esterases. In these molecules, fluorescence resonance energy transfer occurs from the 7-hydroxycoumarin moiety to the fluorescein moiety, leading to green fluorescence when the compounds are excited at about 400 nm. After cleavage of the β -lactam ring, excitation of the 7-hydroxycoumarin moiety results in blue fluorescence; in exemplary embodiments, a 25-fold increase in fluorescence at about 450 nm and a three- to fourfold decrease in fluorescence at 515 nm was observed.

MONITORING GENE EXPRESSION

The substrates of this invention make it feasible to use β -lactamase as a reporter gene to monitor the expression from a set of expression control sequences. In one aspect, this invention provides methods for monitoring gene expression from a set of expression control sequences by using β -lactamase as a reporter gene. A cell is provided that has been transfected with a recombinant nucleic acid molecule comprising the expression control sequences operably linked to nucleic acid sequences coding for the expression of β -lactamase.

Recombinant Nucleic Acids

As used herein, the term "nucleic acid molecule" includes both DNA and RNA molecules. It will be understood that when a nucleic acid molecule is said to have a DNA sequence, this also includes RNA molecules having the corresponding RNA sequence in which "U" replaces "T." The term "recombinant nucleic acid molecule" refers to a nucleic acid molecule which is not naturally occurring, and which comprises two nucleotide sequences which are not naturally joined together. Recombinant nucleic acid molecules are produced by artificial combination, e.g., genetic engineering techniques or chemical synthesis.

Nucleic acids encoding β -lactamases can be obtained by methods known in the art, for example, by polymerase chain reaction of cDNA using primers based on the DNA sequence in Fig. 1. PCR methods are described in, for example, U.S. Pat. No. 4,683,195; Mullis et al. (1987) *Cold Spring Harbor Symp. Quant. Biol.* 51:263; and Erlich, ed., *PCR Technology*, (Stockton Press, NY, 1989).

The construction of expression vectors and the expression of genes in transfected cells involves the use of molecular cloning techniques also well known in the art. Sambrook et al., *Molecular Cloning -- A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, (1989) and *Current Protocols in Molecular Biology*, F.M. Ausubel et al., eds., (Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (most recent Supplement)).

Nucleic acids used to transfect cells with sequences coding for expression of the polypeptide of interest generally will be in the form of an expression vector including expression control sequences operatively linked to a nucleotide sequence coding for expression of the polypeptide. As used, the term nucleotide sequence "coding for expression of" a polypeptide refers to a sequence that, upon transcription and translation of mRNA, produces the polypeptide. As any person skilled in the art recognizes, this includes all degenerate nucleic acid sequences encoding the same amino acid sequence. This can include sequences containing, e.g., introns. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are "operatively linked" to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus, expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (i.e., ATG) in front of a protein-encoding gene, splicing signals for introns, maintenance of

the correct reading frame of that gene to permit proper translation of the mRNA, and stop codons.

The recombinant nucleic acid can be incorporated into an expression vector comprising expression control sequences operatively linked to the recombinant nucleic acid. The expression vector can be adapted for function in prokaryotes or eukaryotes by inclusion of appropriate promoters, replication sequences, markers, etc.

The recombinant nucleic acid used to transfect the cell contains expression control sequences operably linked to a nucleotide sequence encoding a β -lactamase. The β -lactamase encoded can be any known to the art or described herein. This includes, for example, the enzymes shown in Fig. 7.

This invention provides novel recombinant nucleic acid molecules including expression control sequences adapted for function in a non-mammalian eukaryotic cell operably linked to a nucleotide sequence coding for the expression of a cytosolic β -lactamase. As used herein, "cytosolic β -lactamase" refers to a β -lactamase that lacks amino acid sequences for secretion from the cell membrane, e.g., the signal sequence. For example, in the polypeptide of Sequence 1 of Fig. 7, the signal sequence has been replaced with the amino acids Met-Ser. Accordingly, upon expression, this β -lactamase remains within the cell.

This invention provides recombinant nucleic acid molecules including expression control sequences adapted for function in a mammalian eukaryotic cell operably linked to a nucleotide sequence coding for the expression of a β -lactamase.

It is further preferable that the ribosome binding site and nucleotide sequence coding for expression of β -lactamase contain sequences preferred by mammalian cells. Such sequences improve expression of β -lactamase in mammalian cells. Preferred sequences for expression in mammalian cells are described in, for example, Kozak, M., *J. Cell Biol.* 108: 229-241 (1989), referred to herein as "Kozak sequences". The nucleotide sequence for cytosolic β -lactamase in Sequence 3 of

Fig. 7 contains Kozak sequences for the nucleotides -9 to 4 (GGTACCACCATGA).

When used in mammalian cells, the expression control sequences are adapted for function in mammalian cells. The method of this invention is useful to testing expression from any desired set of expression control sequences. In particular, this invention is useful for testing expression from inducible expression control sequences. As used herein, "inducible expression control sequences" refers to expression control sequences which respond to biochemical signals either by increasing or decreasing the expression of sequences to which they are operably linked. For example, in the case of genes induced by steroid hormones, the expression control sequences includes hormone response elements. The binding of a steroid hormone receptor to the response element induces transcription of the gene operably linked to these expression control sequences. Expression control sequences for many genes and for inducible genes, in particular, have been isolated and are well known in the art. The invention also is useful with constitutively active expression control sequences.

The transfected cell is incubated under conditions to be tested for expression of β -lactamase from the expression control sequences. The cell or an extract of the cell is contacted with a β -lactamase substrate of the invention under selected test conditions and for a period of time to allow catalysis of the substrate by any β -lactamase expressed. Then the donor moiety from this sample is excited with appropriate ultraviolet or visible wavelengths. The degree of fluorescence resonance energy transfer in the sample is measured.

If the cell did not express β -lactamase, very little of the substrate will have been cleaved, the efficiency of FRET in the cell will be high, and the fluorescence characteristics of the cell or sample from it will reflect this efficiency. If the cell expressed a large amount of β -lactamase, most of the substrate will be cleaved. In this case, the efficiency of FRET is low, reflecting a large amount

or high efficiency of the cleavage enzyme relative to the rate of synthesis of the tandem fluorescent protein construct. In one aspect, this method can be used to compare mutant cells to identify which ones possess greater or less enzymatic activity. Such cells can be sorted by a fluorescent cell sorter based on fluorescence.

Also, as will be apparent to those working in the field of using reporter gene cell-based assays for screening samples or pools of samples (such as compounds (combinatorial or synthetic), natural product extracts, or marine animal extracts) to identify potential drug candidates which act as agonists, inverse agonists or antagonists of cellular signaling or activation, the combination of cells (preferably mammalian) genetically engineered to express beta-lactamase under the control of different regulatory elements/promoters and the use of the novel beta-lactamase substrate compounds of the present invention will provide distinct advantages over known reporter genes (including, but not limited to, chloramphenicol acetyl transferase, firefly luciferase, bacterial luciferase, Vargula luciferase, aequorin, beta-galactosidase, alkaline phosphatase) and their requisite substrates.

By the choice of appropriate regulatory elements and promoters to control expression of beta-lactamase, assays can be constructed to detect or measure the ability of test substances to evoke or inhibit functional responses of intracellular hormone receptors. These include expression control sequences responsive to inducible by mineralcorticosteroids, including dexamethasone [J. Steroid Biochem. Molec. Biol. Vol. 49, No. 1 1994, pp.31-3]), glucocorticoid, and thyroid hormone receptors [as described in US patent 5,071,773]. Additional such intracellular receptors include retinoids, vitamin D3 and vitamin A [Leukemia vol 8, Suppl. 3, 1994 ppS1-S10; Nature Vol. 374, 1995, p.118-119; Seminars in Cell Biol., Vol. 5, 1994, p.95-103]. Specificity would be enabled by use of the appropriate promoter/enhancer element. Additionally, by choice of other regulatory elements or specific promoters, drugs which

influence expression of specific genes can be identified. Such drugs could act on specific signaling molecules such as kinases, transcription factors, or molecules such signal transducers and activators of transcription [Science Vol. 264, 1994, p.1415-1421; Mol. Cell Biol., Vol. 16, 1996, p.369-375]. Specific microbial or viral promoters which are potential drug targets can also be assayed in such test systems.

Also by the choice of promoters such as c-fos or c-jun [US patent 5,436,128; Proc. Natl. Acad. Sci. Vol. 88, 1991, pp. 5665-5669] or promoter constructs containing regulatory elements responsive to second messengers [Oncogene, 6: 745-751 (1991)] (including cyclic AMP-responsive elements, phorbol ester response element (responsive to protein kinase C activation), serum response element (responsive to protein kinase C-dependent and independent pathways) and Nuclear Factor of Activated T-cells response element (responsive to calcium) to control expression of beta-lactamase, assays can be constructed to detect or measure substances or mixtures of substances that modulate cell-surface receptors including, but not limited to, the following classes: receptors of the cytokine superfamily such as erythropoietin, growth hormone, interferons, and interleukins (other than IL-8) and colony-stimulating factors; G-protein coupled receptors [US patent 5,436,128] for hormones, such as calcitonin, epinephrine or gastrin, pancrine or autocrine mediators, such as somatostatin or prostaglandins, and neurotransmitters such as norepinephrine, dopamine, serotonin or acetylcholine; tyrosine kinase receptors such as insulin growth factor, nerve growth factor [US patent 5,436,128]. Furthermore, assays can be constructed to identify substances that modulate the activity of voltage-gated or ligand-gated ion channels, modulation of which alters the cellular concentration of second messengers, particularly calcium [US patent 5,436,128]. Assays can be constructed using cells that intrinsically express the promoter, receptor or ion channel of interest or into which the appropriate protein has been genetically engineered.

The expression control sequences also can be those responsive to substances that modulate cell-surface receptors or that modulate intra-cellular receptors.

To measure whether a substance or mixture of substances activates extracellular or intracellular receptors or other cellular responses, cells containing beta-lactamase controlled by a desired promoter/enhancer element are incubated with test substance(s), substrate then added, and after a certain period of time the fluorescence signal is measured at either one or two excitation-emission pairs appropriate to the chosen compound of the invention (e.g. compound CCF2 with wavelength pairs of near 405 nm and near 450 nm and near 405 and near 510 nm). This fluorescent result is compared to control samples which have had no drug treatment and, when feasible, control samples with a known inhibitor and a known activator. The effect of any active drugs is then determined using the ratio of the fluorescence signal found in test wells to the signals found in wells with no drug treatment. Assays are performed in wells in a microtiter plate containing 96 or more wells or in an assay system with no compartments such as a gel matrix or moist membrane environment. Detection could be done for example by microtiter plate fluorimeters, e.g. Millipore Cytofluor, or imaging devices capable of analyzing one or more wells or one or more assay points in a certain surface area, e.g. as supplied by Astromed. The ability to retain the substrate in the cytoplasm of living cells is advantageous as it can allow a reduction in signal interference from coloured or quenching substances in the assay medium. Furthermore, the fluorescent signal from the compounds of this invention, such as CCF2, can be readily detected in single cells and thus allowing assay miniaturization and an increased number of tests per surface area. Miniaturized assays also further increase the throughput of an imaging detection system as there are more samples within the imaging field.

The assay systems of the present invention further provide an advantageous and rapid method of isolation and clonal selection of stably transfected cell lines containing

reporter genes and having the desired properties which the transfection was intended to confer, e.g. fluorescent signal response after activation of a transfected receptor with a high signal-to-noise ratio of at least 10:1 from a high proportion of isolated cells. Current procedures for clonal selection of satisfactorily transfected, genetically engineered cells from the population initially transfected with the vectors of interest, are done mainly by manual means and involve several rounds of microscopic analyses, selecting the visually preferred clone, isolation of the clone by manual pipetting stages and prolonged cellular cultivations. This procedure is laborious and time-consuming; it may require several months to generate a clone useful for assays suited to drug screening. Moreover, it is difficult to manually select and maintain more than a few hundred clones. Using the assays of this present invention, the desired signal from cellular beta-lactamase reporter system can be maintained within living and viable cells. Thus, one can rapidly select, from the population of initially transfected cells, those few living cells with the best fluorescent signal using automated instruments such as a fluorescent-activated cell sorter, e.g. the Becton Dickinson FACS Vantage. The selected cells are then collected for cultivation and propagation to produce a clonal cell line with the desired properties for assays and drug screening.

In addition, the presence (for example, in human serum, pus or urine) of bacteria resistant to β -lactam antibiotics can be readily detected using the substrates of the present invention. Only in the presence of an active β -lactamase is there a change in the fluorescence spectrum from that of the intact molecule to one characteristic of the cleavage product. The substrates of the present invention are superior to prior art chromogenic substrates Nitrocephin and PADAC, in that the inventive substrates are stable to human serum. The novel substrates are also more sensitive than the chromogenic substrate CENTA, because they experience a much smaller optical background signal from human serum and a lower detection limit for fluorescence versus absorbance.

The invention may be better understood with reference to the accompanying examples, which are intended for purposes of illustration only and should not be construed as in any sense limiting the scope of the invention as defined in the claims appended hereto.

MEASUREMENTS

The degree of FRET can be determined by any spectral or fluorescence lifetime characteristic of the excited construct, for example, by determining the intensity of the fluorescent signal from the donor, the intensity of fluorescent signal from the acceptor, the ratio of the fluorescence amplitudes near the acceptor's emission maxima to the fluorescence amplitudes near the donor's emission maximum, or the excited state lifetime of the donor. For example, cleavage of the linker increases the intensity of fluorescence from the donor, decreases the intensity of fluorescence from the acceptor, decreases the ratio of fluorescence amplitudes from the acceptor to that from the donor, and increases the excited state lifetime of the donor.

Preferably, changes in the degree of FRET are determined as a function of the change in the ratio of the amount of fluorescence from the donor and acceptor moieties, a process referred to as "ratioing." Changes in the absolute amount of substrate, excitation intensity, and turbidity or other background absorbances in the sample at the excitation wavelength affect the intensities of fluorescence from both the donor and acceptor approximately in parallel. Therefore the ratio of the two emission intensities is a more robust and preferred measure of cleavage than either intensity alone.

The excitation state lifetime of the donor moiety is, likewise, independent of the absolute amount of substrate, excitation intensity, or turbidity or other background absorbances. Its measurement requires equipment with nanosecond time resolution, except in the special case of lanthanide complexes in which case microsecond to millisecond resolution is sufficient.

Additional suitable Chel moieties are described in Vallarino, L.M., & Leif, R.C., US Pat. 5,373,093; Sabbatini, N. et al, *Pure and Applied Chem.* 67: 135-140 (1995); Mathis, G., *Clinical Chem.* 41: 1391-1397 (1995); Horiguchi, D., *Chem. Pharm. Bull.* 42: 972-975 (1994); Takalo, H. et al, *Bioconjugate Chem.* 5: 278-282 (1994); Saha, A.K. et al, *J. Amer. Chem. Soc.* 115: 11032 (1993); Li, M. & Selvin, P.R., *J. Amer. Chem. Soc.* 117: 8132-8138 (1995).

Fluorescence in a sample is measured using a fluorimeter. In general, excitation radiation, from an excitation source having a first wavelength, passes through excitation optics. The excitation optics cause the excitation radiation to excite the sample. In response, fluorescent proteins in the sample emit radiation which has a wavelength that is different from the excitation wavelength. Collection optics then collect the emission from the sample. The device can include a temperature controller to maintain the sample at a specific temperature while it is being scanned. According to one embodiment, a multi-axis translation stage moves a microtiter plate holding a plurality of samples in order to position different wells to be exposed. The multi-axis translation stage, temperature controller, auto-focusing feature, and electronics associated with imaging and data collection can be managed by an appropriately programmed digital computer. The computer also can transform the data collected during the assay into another format for presentation.

Methods of performing assays on fluorescent materials are well known in the art and are described in, e.g., Lakowicz, J.R., *Principles of Fluorescence Spectroscopy*, New York: Plenum Press (1983); Herman, B., Resonance energy transfer microscopy, in: *Fluorescence Microscopy of Living Cells in Culture, Part B, Methods in Cell Biology*, vol. 30, ed. Taylor, D.L. & Wang, Y.-L., San Diego: Academic Press (1989), pp. 219-243; Turro, N.J., *Modern Molecular Photochemistry*, Menlo Park: Benjamin/Cummings Publishing Co., Inc. (1978), pp. 296-361.

EXAMPLES

All silica gel chromatography was performed using silica gel (Merck, grade 60, 230-400 mesh, 60Å) purchased from Aldrich. Bakerbond Octadecyl from J. T. Baker was used for C₁₈ reverse phase chromatography. Solvents (high pressure liquid chromatography grade) were used for chromatography as received, or dried over activated molecular sieves (3 Å) for synthetic purposes.

Fluorescence excitation and emission spectra were measured either on a Spex Fluorolog 111 or on a K2 fluorometer (ISS, Champaign, IL) in ratio mode with a rhodamine B quantum counter. The efficiency of fluorescence energy transfer was determined from the change in the integrated fluorescence emission at the donor emission wavelength upon treatment with β-lactamase. For fluorescence microscopy imaging, two different imaging setups were used. One, with an inverted fluorescence microscope, Zeiss IM-35 (Thornwood, NY) coupled to a silicon-intensified target (SIT) camera (Dage-MTI, Michigan City, IN) has been described in detail [Tsien, R.Y. (1986) New tetracarboxylate chelators for fluorescence measurement and photochemical manipulation of cytosolic free calcium concentrations, in: *Optical Methods in Cell Physiology*, ed. de Weer, P. & Salzberg, B., New York:Wiley, pp. 327-345; Tsien and Harootunian (1990) *Cell Calcium* 11:93-109]. The other consisted of a cooled charge-coupled-device (CCD) camera (Photometrics, Tucson, AZ) connected to an inverted fluorescence microscope (Zeiss Axiovert).

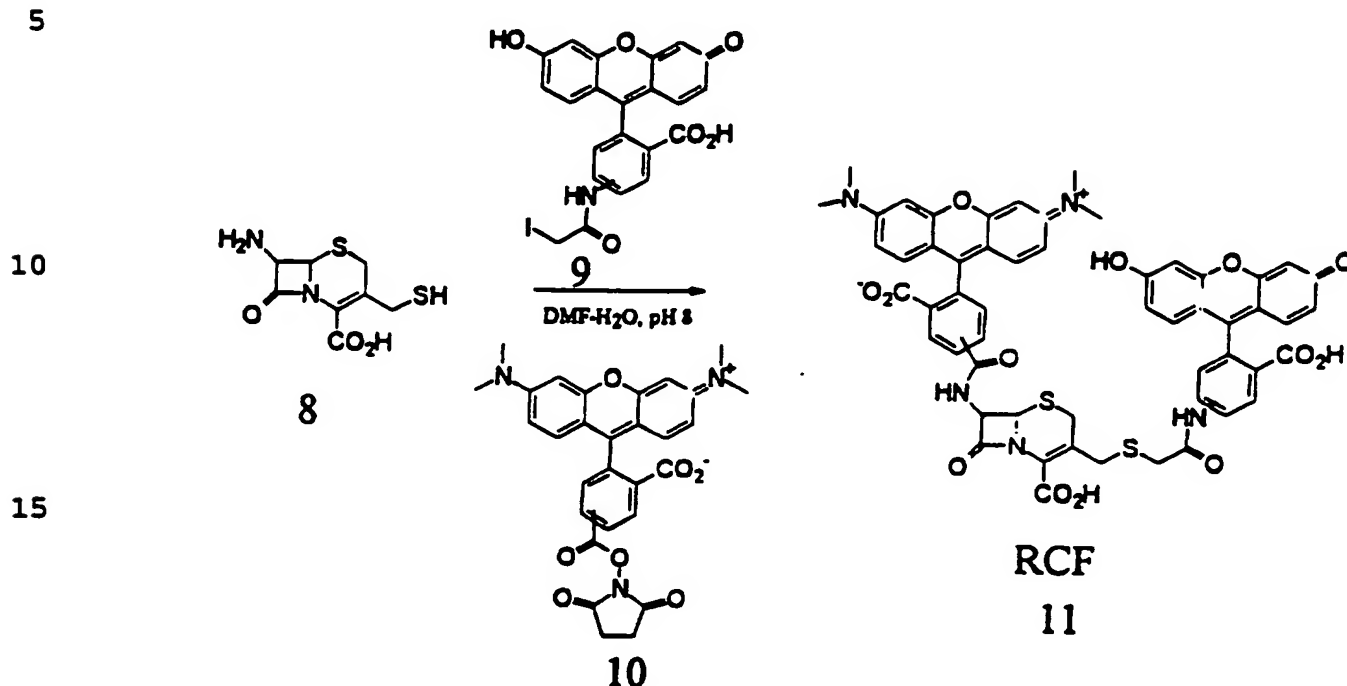
Fluorescence resonance energy transfer was measured by monitoring the ratio of fluorescence intensities at donor and acceptor emission wavelengths using commercially-available filters (Omega Optical).

Excitation: 360 DF 40, dichroic mirror 390 DCLP
or 405 DF 15, dichroic mirror 420 DRLPO2

Emission: 450 DF 65 (donor emission)
515 EFLP (acceptor emission)
435 EFLP (to view donor and acceptor
fluorescence simultaneously)

Example 1 (Compound 11)

To test the optical properties of a cephalosporin with two dye molecules attached the following model compound was synthesized.



20 The first synthesis step was to convert 7-aminocephalosporanic acid into a bifunctional cephalosporin carrying a thiol in the 3'-position and the 7-amine [Van Heyningen, E. and Brown, C.N., *J. Med. Chem.* 8: 174-181 (1965); Japanese Patent, Kokai 75/18494, CA 85, 97320d]. This cephalosporin was then reacted

25 selectively with an thiol-reactive dye, followed by an amine-reactive dye. The thiol-reactive dye 5, (6)-iodoacetamido-fluorescein and the amine-reactive dye 5, (6)-carboxy-N,N,N',N'-tetramethylrhodamine-succinimide were coupled to the cephalosporin in aqueous dimethylformamide at pH 8. The

30 product will be referred to as RCF.

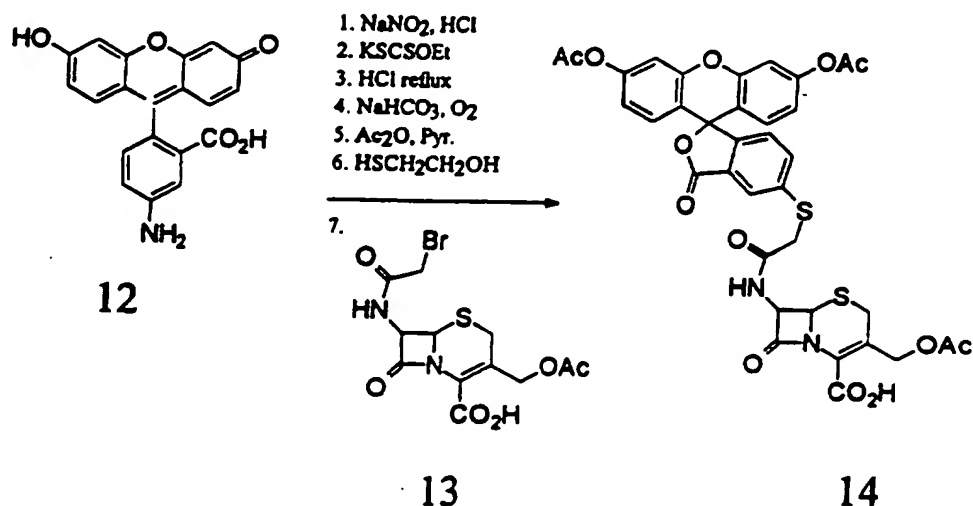
In phosphate buffer at pH 7 RCF is virtually non fluorescent; neither fluorescein nor rhodamine show much fluorescence when excited at their respective excitation maxima, which is indicative of chromophore stacking ("dark complex"). After long term treatment with β -lactamase the β -lactam is cleaved causing the fluorescence of both dyes to

35 reappear (Figs. 1(a) and 1(b)). This experiment confirms that one can measure β -lactamase catalyzed hydrolysis of the

β -lactam in cephalosporins by the loss of fluorescence quenching using an appropriate donor-acceptor pair.

Example 2

The thiomethyl linker was introduced by conversion of 5-fluoresceinamine to 5-mercaptofluorescein via diazotization, conversion to the ethylxanthate, and degradation of the xanthate by aqueous acid to the free sulfhydryl. It was coupled to 7-bromoacetamido-
cephalosporanic acid by nucleophilic displacement of the bromide by the mercapto group of the fluorescein. 7-Bromoacetamido-cephalosporanic acid had been prepared from 7-aminocephalosporanic acid and bromoacetyl bromide [Bunnell, C.A. et al. Industrial manufacture of cephalosporins. In: *Beta-Lactam Antibiotics for Clinical Use. Series: Clinical Pharmacology Vol. 4*, edited by Queener, S.F., Webber, J.A. and Queener, S.W. New York: M. Dekker, 1986, p. 255-283].

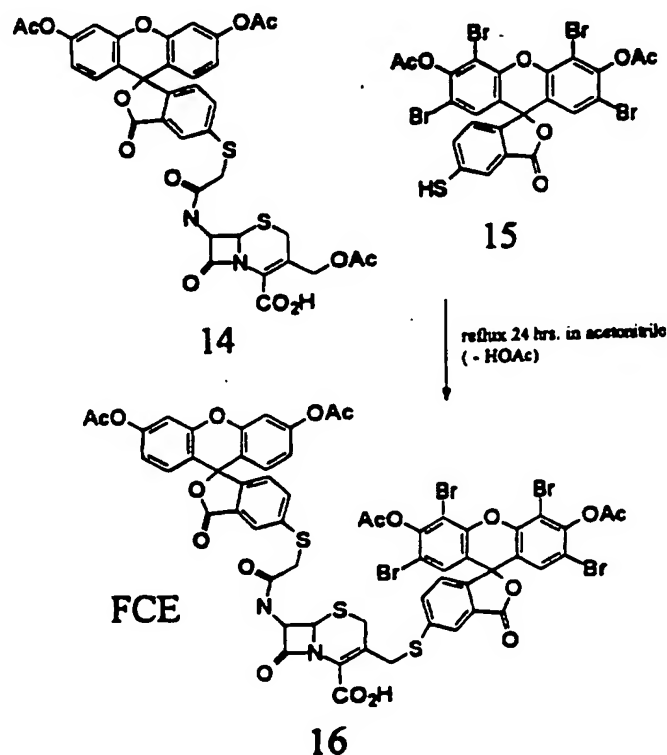


To prepare

7β -[(5-diacetylfluorescein)thio]acetamido-3-(acetoxymethyl)-3-cephem-4-carboxylic acid (14), in a nitrogen atmosphere 130mg (0.29mmol) 5-fluoresceinthiol diacetate were dissolved in 10ml dimethylformamide and added to 120mg (0.31mmol) 7β -bromoacetamido-3-(acetoxymethyl)-3-cephem-4-carboxylic acid

in 10ml 1M potassium phosphate buffer adjusted to pH 8.0. The solution was stirred for 8 hours at room temperature after which the solvents were removed in vacuo. The residue was dissolved in 10ml water and the pH of the solution was carefully adjusted to pH 5 with dilute phosphoric acid. At this point nonpolar byproducts precipitated and were removed by centrifugation. Further acidification to pH 2.7 precipitated the title compound which was collected by centrifugation, washed 3 times with 2 ml diethylether-tetrachloromethane (1:2), and dried in vacuo. ¹H NMR (CDCl₃): δ 2.08ppm (s, 3H, acetate), δ 3.36ppm, 3.53ppm (2d, 2H, J=17.3 Hz, C-2), δ 3.87ppm (s, 2H, side chain methylene), δ 4.88ppm, 5.16ppm (2d, 2H, J=13.6 Hz, C-3'), δ 4.96ppm (d, 1H, J=4.9 Hz, C-6), δ 5.81ppm (dd, 1H, J₁=8.2 Hz, J₂=4.9 Hz, C-7), δ 6.85ppm (m, 4H, xanthene), δ 7.10 (s, 2H, xanthene), δ 7.15ppm (d, 1H, J=8.2 Hz, amide), δ 7.69ppm (d, 1H, J=8.2 Hz, phthalic), δ 7.91ppm (d, 1H, J=8.2 Hz, phthalic), δ 8.11ppm (s, 1H, phthalic).

5-Fluoresceinamine was brominated to generate 5-eosinamine, which was converted into 5-mercaptos eosin in analogous way to the 5-mercaptofluorescein. In a nucleophilic displacement of the cephalosporin acetate by 5-mercaptos eosin diacetate the FRET-cephalosporin was generated as the protected tetraacetyl derivative.



To prepare 5-eosinamine, 1.74g (5mmol)

5-fluoresceinamine was suspended in 30ml glacial acetic and 2.06ml (40mmol, 100% excess) bromine was added. With the addition of bromine the fluoresceinamine went into solution.

5 The solution was heated for six hours at 90°C, during which period a white precipitate began to form. An ice-cooled trap attached to the flask kept bromine from escaping into the atmosphere. Excess bromine was then recovered by distillation

10 into a liquid nitrogen cooled collecting flask. One volume of water was added to the acetic acid solution to precipitate any product remaining in solution. The precipitate was collected by filtration and dissolved in 1N aqueous sodium hydroxide.

5-Eosinamine was precipitated as the free amine by addition of glacial acetic acid. The eosinamine was dissolved in little
15 chloroform and methanol was added. Concentrating this solution on the rotary evaporator gave 2.56g (3.85mmol, 77%) eosinamine as a fine white powder (the eosinamine-spirolactone).

To prepare 5-eosin-ethylxanthate diacetate, 670mg
20 (1mmol) 5-eosinamine were stirred in 2ml concentrated sulfuric acid and 2ml glacial acetic acid. The suspension was cooled with an ice-salt bath to a few degrees below 0°C, which turned it into a thick paste that was difficult to stir. 200mg (2.9mmol) sodium nitrite in 1ml water were added dropwise over
25 the period of one hour. After another 2 hours at 0°C 20g of ice was slowly added. The flask was put on the high vacuum pump in the cold, to remove excess nitrous gases (caution!!). Saturated ice-cold aqueous sodium bicarbonate solution was added until the solids dissolved into the dark red solution.
30 200mg (1.2mmol) Potassium ethylxanthate was added and a pink precipitate formed (5-eosindiazonium xanthate). A few crystals of nickel(II)chloride catalyzed the conversion of the diazonium salt with evolution of nitrogen. Once nitrogen evolution had ceased the products were precipitated with 1N
35 hydrochloric acid. The precipitate was collected by filtration and dried in vacuo. It was treated with acetic anhydride-pyridine (1:1) at 40°C for one hour. After removal of the reagents in vacuo, the residue was chromatographed over

silica gel with ethyl acetate-hexane (1:4) as eluent. The desired product eluted first. The yield was 110mg (0.13mmol, 13%) of the title compound as a white powder.

For preparation of the disulfide dimer of
5 5-eosinithiol diacetate (dimer of 15), 110mg (0.13mmol)
5-eosin-ethylxanthate diacetate was stirred in 10ml
concentrated (30%) aqueous ammonia and the solution was heated
to 70°C. Air was bubbled slowly through the solution to
oxidize the thiol to the disulfide *in situ*. After 2 hours the
10 solvents were removed on the rotary evaporator at 40°C and the
residue was treated with acetic anhydride-pyridine (1:1).
After removal of the reagents *in vacuo* the residue was
chromatographed over silica gel with ethyl acetate-hexane
(1:4) as the eluent. Yield was 90mg (60μmol, 91%) of the
15 title product as a white powder. The compound was reduced to
the monomer (15) by dissolving it in methanol with addition of
sodium acetate and addition of 20 equivalents mercaptoethanol.
After 2 hours the methanolic solution was poured into 3
volumes 5% aqueous acetic acid from which the precipitating
20 5-fluoresceinithiol monomer was collected by centrifugation.
The solid was washed with water until no odor of
mercaptoethanol remained.

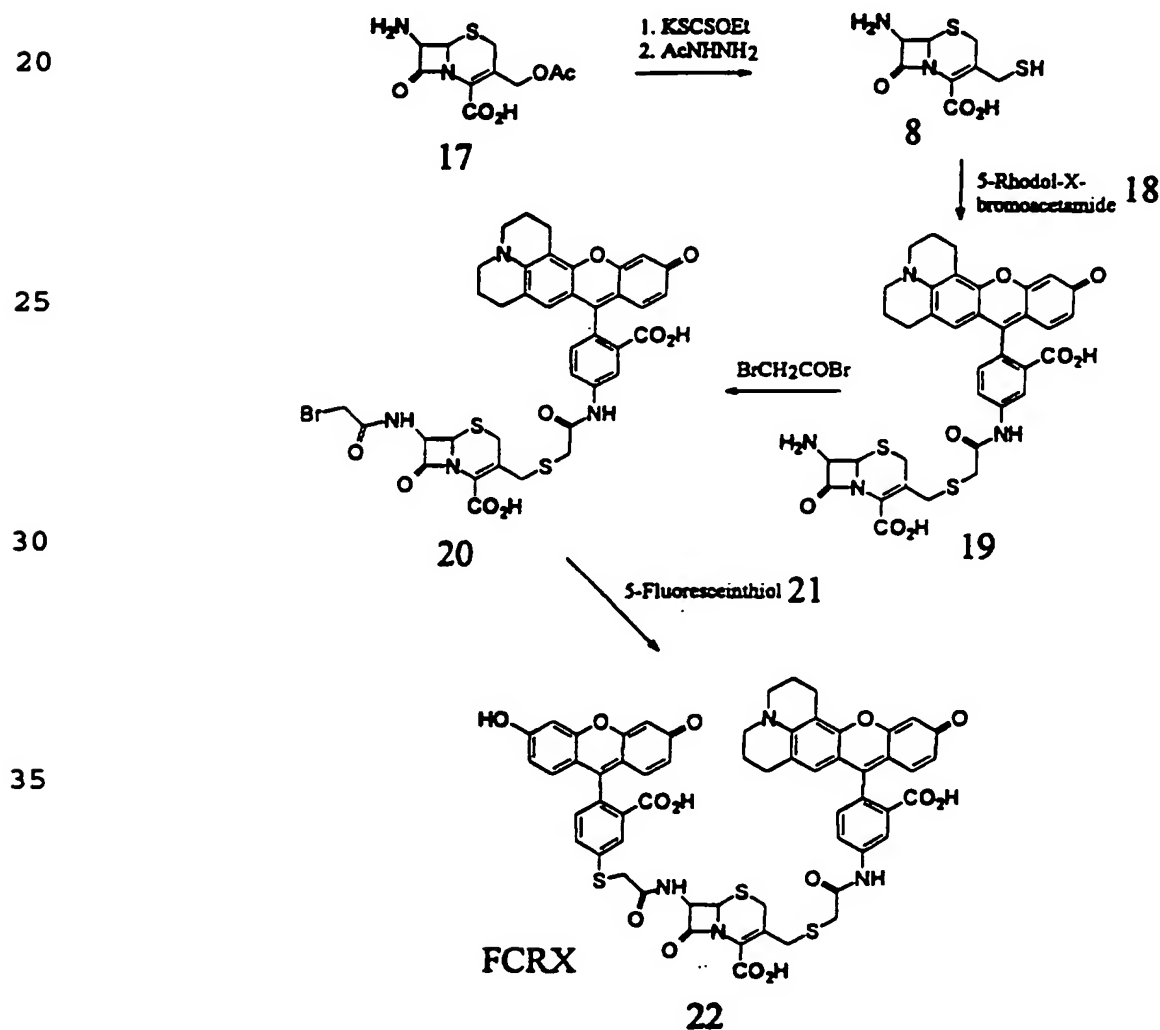
Coupling of diacetyl 5-eosinithiol (15) with
7β-[(5-diacetylfluorescein)thio]acetamido-3-(acetoxymethyl)-
25 3-cephem-4-carboxylic acid (14) and deacylation with
acetylcysteine was effected as follows. 10mg (13μmol)
7β-[(5-Diacetylfluorescein)thio]acetamido-3-(acetoxymethyl)-
3-cephem-4-carboxylic acid and 10mg (13μmol) diacetyl-
5-eosinithiol were dissolved in 200μl dry acetonitrile and the
30 solution was sealed under argon in a glass tube. The tube was
kept in an oil bath at 84°C (± 2°C) for 16 hours. Then it was
cut open, the solution transferred to a flask and the solvent
removed *in vacuo*. The residue was flash-chromatographed over
silica gel with ethyl acetate-methanol-acetic acid (100:1:1)
35 as the eluent. Deprotection of the acetates was achieved by
incubating the product with orange peel acetylcysteine in 50mM
phosphate buffer (pH7) for 24 hours at 37°C. The deacylated
product was purified by C₁₈ reverse phase chromatography. The

eluent was a step gradient of 25mM aqueous phosphate buffer (pH7) and methanol. Fluorescein byproducts eluted with 33% and 50% methanol in the eluent, after which the desired product eluted in 66% methanol.

5 The deprotected compound shows little fluorescence in phosphate buffer as the two hydrophobic dyes stack. The remaining fluorescence is due to fluorescence resonance energy transfer (FRET). This compound is a good substrate for RTEM β -lactamase and will be referred to as FCE.

10 Cleavage of the compound increases fluorescence at 515nm about 70-fold (Fig. 2). The fluorescence properties of the compound can be attributed to dye-dimer formation, as FRET increases drastically once methanol is added to the solution. Methanol breaks the hydrophobic interaction that causes the
15 fluorophores to stack.

Example 3 (compound 22)



The 3'-acetate of 7-aminocephalosporanic acid was displaced by ethylxanthate [Van Heyningen and Brown (1965), supra] which was hydrolysed to the free sulfhydryl with aqueous acetylhydrazine [Japanese Patent, Kokai 75/18494, CA85, 97320d]. The sulfhydryl group was reacted with 5-bromoacetamido-rhodol-X in aqueous dimethylformamide. The cephalosporin 7-amine was reacted with bromoacetyl bromide in aqueous dioxane, followed by bromide displacement with 5-fluoresceinthiol to yield a FRET-cephalosporin that is virtually nonfluorescent in 50mM phosphate buffer pH 7. This compound is referred to as FCRX.

The first step in preparation of 5-rhodol-X-bromoacetamide was synthesis of 9-(2'-carboxy-4'(5')-nitro-benzoyl)-8-hydroxyjulolidine and separation of the isomers. 10.1g (48mmol, 92% purity) 4-Nitrophthalic anhydride were dissolved in 20ml toluene at 70°C. 9.76g (50mmol, 97% purity) 8-Hydroxyjulolidine in 20ml ethyl acetate were added and the solution kept at 70°C for 30min. The reaction mixture was run through a short bed of silica gel followed by ethyl acetate as eluent. The solvents were removed in vacuo and the solid redissolved in a minimum amount of refluxing ethyl acetate. The isomer with the nitro-group meta to the benzoic acid crystallizes over night from solution in orange crystals (3.47g in first fraction). After additional fractional crystallization the pure isomer was obtained. ¹H NMR (CDCl₃) of crystallized isomer: δ 1.91ppm (m, 4H, aliphatic methylenes), δ 2.73ppm, 2.46ppm (2m, 4H, anilinic methylenes), δ 3.26ppm (m, 4H, benzylic methylenes), δ 6.32ppm (s, 1H, julolidine), δ 7.53ppm (d, 1H, J=8.4 Hz, phthalic), δ 8.43ppm (dd, J₁=8.4 Hz, J₂=2.2 Hz, phthalic), δ 8.90ppm (d, 1H, J=2.2 Hz, phthalic).

For preparation of 5-rhodol-X-amine hydrochloride (named by analogy with rhodamine-X), 1.91g (5.0mmol) 9-(2'-Carboxy-4'-nitro-benzoyl)-8-hydroxyjulolidine was stirred in 5ml concentrated (96%) sulfuric acid. 700mg (6.4mmol, 1.25equ.) resorcinol was added with cooling over a period of 15 minutes. The suspension was stirred 1.5 hours at room temperature and then poured into 200ml water with

vigorous stirring. The purple precipitate was collected by filtration and redissolved in 75ml water with the help of 5.3g (22mmol) sodium sulfide nonahydrate. 2.5g (44.6 mmol) Anhydrous sodium bisulfide was added and the solution refluxed for 24 hours. Then, after cooling to room temperature, the product was precipitated by addition of glacial acetic acid. The solid was collected by filtration and boiled with 100ml half-saturated aqueous hydrochloric acid. The solution was filtered hot through a glass frit to remove sulfur. The solution volume was reduced to 10ml on the rotary evaporator. 1 Volume saturated brine was added and the precipitate collected by filtration. Crystallization from refluxing hydrochloric acid yielded 1.78g (3.85mmol, 77%) dark red crystals of 5-rhodol-X-amine hydrochloride. ¹H NMR (dDMSO) of 5-nitro-rhodol-X: δ 1.90ppm, 2.05ppm (2m, 4H, aliphatic methylenes), δ 2.72ppm, 3.03ppm (2m, 4H, anilinic methylenes), δ 3.66ppm (m, 4H, benzylic methylenes), δ 6.90ppm (s, 1H, xanthene), δ 6.96ppm (dd, 1H, $J_1=9.0$ Hz, $J_2=2.1$ Hz, xanthene), δ 7.11ppm (d, 1H, $J=9.0$ Hz, xanthene), δ 7.22ppm (d, 1H, $J=2.1$ Hz, xanthene), δ 7.78ppm (d, 1H, $J=8.4$ Hz, phthalic), δ 8.70ppm (dd, 1H, $J_1=8.4$ Hz, $J_2=2.4$ Hz, phthalic), δ 8.91ppm (d, 1H, $J=2.4$ Hz, phthalic). ¹H NMR (CD₃OD) of 5-rhodol-X-amine hydrochloride: δ 2.00ppm, 2.14ppm (2m, 4H, aliphatic methylenes), δ 2.75ppm, 3.11ppm (2m, 4H, anilinic methylenes), δ 3.67ppm (m, 4H, benzylic methylenes), δ 6.85ppm (s, 1H, xanthene), δ 6.94ppm (dd, 1H, $J_1=9.0$ Hz, $J_2=2.1$ Hz, xanthene), δ 7.13ppm (d, 1H, $J=9.0$ Hz, xanthene), δ 7.16ppm (d, 1H, $J=2.1$ Hz, xanthene), δ 7.55ppm (d, 1H, $J=8.1$ Hz, phthalic), δ 7.82ppm (dd, 1H, $J_1=8.1$ Hz, $J_2=1.9$ Hz, phthalic), δ 8.28ppm (d, 1H, $J=1.9$ Hz, phthalic).

Preparation of 5-rhodol-X-bromoacetamide (18) was effected as follows. 115mg (0.25mmol) 5-Rhodol-X-amine hydrochloride were dissolved with 180mg (2.1mmol) sodium bicarbonate in 2ml water-dioxane (1:1). The solution was cooled on ice and 175 μ l (2mmol) bromoacetyl bromide were added with stirring over a period of 20 minutes. The solution was then kept at room temperature for 1.5 hours, after which 5 volumes of water were added. The dioxane was removed on the

rotary evaporator, and the product was precipitated from the remaining aqueous solution by addition of acetic acid. The precipitate was filtered off and dissolved in a small volume of chloroform-methanol (1:1). Silica gel was added to the solution and the solvents removed in vacuo. The solids were applied to a silica gel column and the product eluted with methanol-ethyl acetate (1:4). This eluent did dissolve some silica gel which remained with the eluted product. ¹H NMR (CD₃OD, 10% dDMSO): δ 1.98ppm, 2.12ppm (2m, 4H, aliphatic methylenes), δ 2.72ppm, 3.06ppm (2m, 4H, anilinic methylenes), δ 3.56ppm (m, 4H, benzylic methylenes), δ 4.08ppm (s, 2H, bromoacetyl), δ 6.79ppm (dd, 1H, J₁=9.2 Hz, J₂=2.1 Hz, xanthene), δ 6.83ppm (s, 1H, xanthene), δ 6.90ppm (d, 1H, J=2.1 Hz, xanthene), δ 7.19ppm (d, 1H, J=9.2 Hz, xanthene), δ 7.24ppm (d, 1H, J=8.4 Hz, phthalic), δ 8.02ppm (dd, 1H, J₁=8.4 Hz, J₂=1 Hz, phthalic), δ 8.30ppm (d, 1H, J=1 Hz, phthalic).

For preparation of 7β-(bromoacetamido)-3-[[[(5-rhodol-X-amido)methyl]thio]methyl]-3-cephem-4-carboxylic acid (20), 4.5mg (10μmol) 5-Rhodol-X-bromoacetamide (18) were dissolved in 0.5ml 250mM phosphate buffer adjusted to pH 7.7 and 0.5ml dimethylformamide. The solution was deoxygenated and 10mg (40μmol) 7β-amino-3-(thiomethyl)-3-cephem-4-carboxylic acid (8) prepared according to the literature procedure in 100μl phosphate buffer was added in an argon atmosphere. The solution was kept for 2 hours at 30°C. Then the solvents were removed in vacuo and the residue dissolved in 1 ml water, from which the product was precipitated by addition of acetic acid. The precipitate was collected and the product purified by C₁₈ reverse-phase chromatography with 0.1% trifluoroacetic acid in 35% methanol/water as eluent.

The above product (19) was dissolved in 1ml dioxane-water (1:1) with 20mg sodium bicarbonate. 10μl Bromoacetyl bromide were added to the solution on ice. The solution was kept for another 1.5 hours at room temperature. 20mg sodium bicarbonate and 10μl bromoacetyl bromide were added to the solution with ice cooling. After another 1.5 hours at room

temperature the dioxane was removed on the rotary evaporator and the products were precipitated from the aqueous solution with 1M phosphoric acid and collected by centrifugation. The solids were suspended in dilute aqueous bicarbonate solution and the undissolved particles removed by centrifugation and discarded. The product was precipitated with 1M phosphoric acid and purified by flash chromatography on silica gel with chloroform-methanol-acetic acid-water (55:15:4:2). This procedure dissolved small amounts of silica gel.

Coupling of diacetyl 5-fluoresceinthiol (21) with 7β -(bromoacetamido)-3-[[[(5-rhodol-X-amido)methyl]thio]methyl]-3-cephem-4-carboxylic acid (20) was effected as follows. 7β -(Bromoacetamido)-3-[[[(5-rhodol-X-amido)methyl]thio]methyl]-3-cephem-4-carboxylic acid was reacted with a 50% excess of 5-fluoresceinthiol under argon with dimethylformamide-(250mM aqueous phosphate buffer pH 7.7) (1:1) as the solvent. The product was purified from excess fluoresceinthiol by repeated dissolution in methanol and precipitation in ethyl acetate.

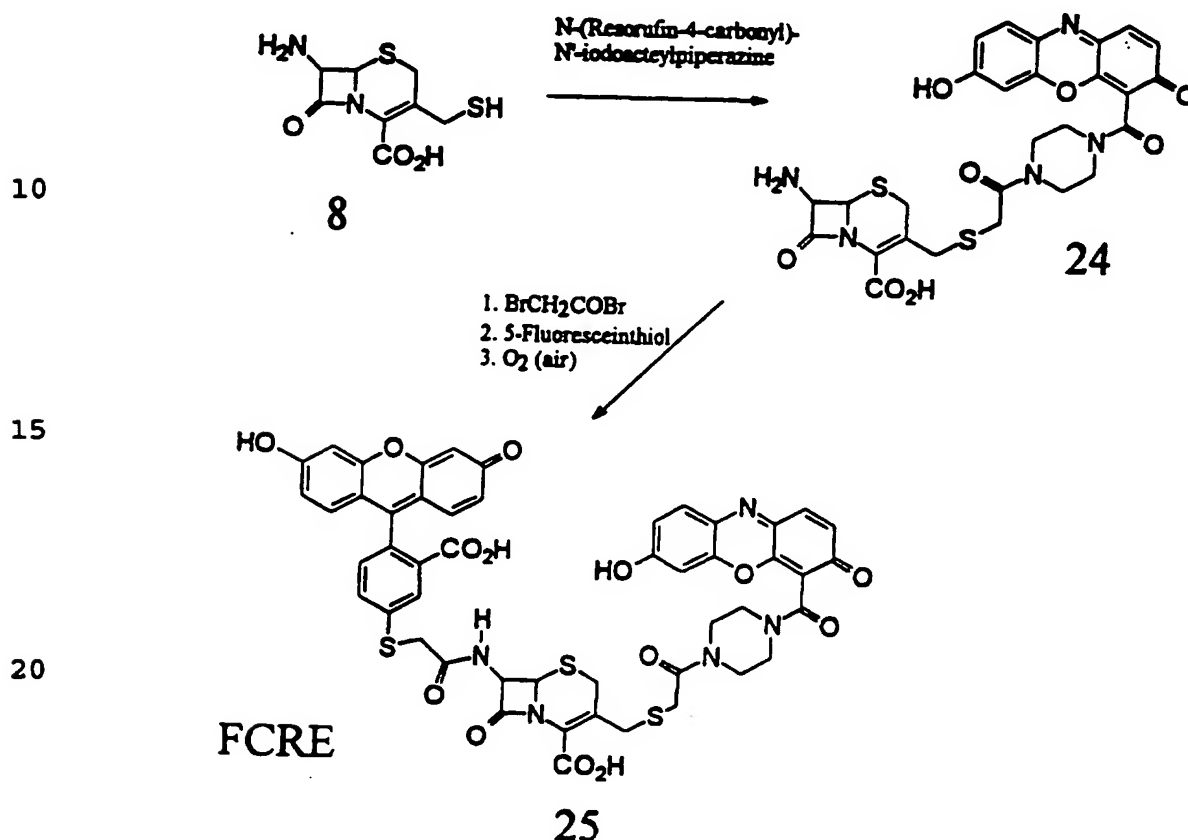
Fig. 3 shows the fluorescence emission spectra of this FRET-cephalosporin in 50mM phosphate buffer pH 7 before and after treatment with β -lactamase. The low initial fluorescence is due to the stacking of the fluorophores, forming a ground state complex that is nonfluorescent. When one adds methanol to the solution this stacking can be disrupted and efficient fluorescence resonance energy transfer occurs.

Example 4 (compound 25)

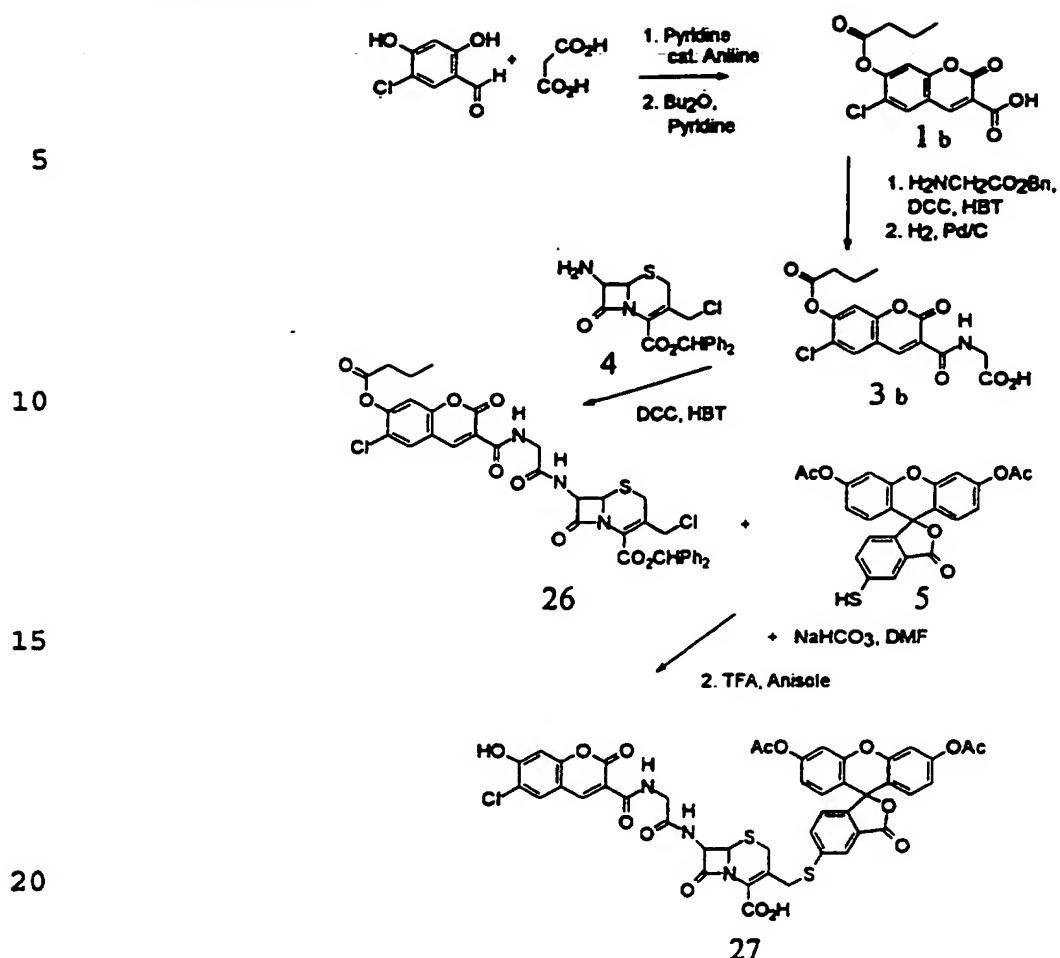
N-[resorufin-4-carbonyl]-N'-iodoacetyl-piperazine (Boehringer Mannheim) was attached to the cephalosporin as a FRET-acceptor for fluorescein. It is referred to as FCRE.

The FRET-cephalosporin FCRE (25) carrying fluorescein as the donor and resorufin as the quencher was made by the same procedure as the one carrying the rhodol-X-acceptor. The N-[resorufin-4-carbonyl]-N'-iodoacetyl-piperazine (Boehringer Mannheim) was coupled to the free 3'-thiol of the cephalosporin followed by bromoacetylation and

addition of the 5-fluoresceinthiol. In departure from the protocol, three equivalents of 5-fluorescein thiol were added, as the first equivalent instantaneously reduced the resorufin and formed unreactive difluorescein-disulfide. Exposure to air reoxidized resorufin to the original dye.



β -Lactamase catalyzed hydrolysis of this compound generates two fluorescent fragments. Resorufin excitation and emission spectra are longer wavelength and narrower than the rhodol spectra, possibly affording better spectral separation between the uncleaved dye versus the products of enzymatic cleavage. But, as in the case of rhodol as the acceptor, in aqueous phosphate buffer the dyes stack and form a dark complex. β -Lactamase treatment disrupts the stacking and increases donor fluorescence (Fig. 4).

Example 5 (compound 7h)

For synthesis of 2,4 dihydroxy-5-chlorobenzaldehyde, 21.7 g (0.15 Mol) 4-chlororesorcinol were dissolved in 150 ml dry diethyl ether and 27 g finely powdered zinc (II) cyanide and 0.5 g potassium chloride were added with stirring. The suspension was cooled on ice. A strong stream of hydrogen chloride gas was blown into the solution with vigorous stirring. After approximately 30 minutes the reactants were dissolved. The addition of hydrogen chloride gas was continued until it stopped being absorbed in the ether solution (approx. 1 hour). During this time a precipitate formed. The suspension was stirred for one additional hour on ice. Then the solid was let to settle. The ethereal solution was poured from the solid. The solid was treated with 100 g of ice and heated to 100°C in a water bath. Upon cooling the product crystallized in shiny plates from the solution. They were removed by filtration on dried over potassium hydroxide.

The yield was 15.9 g (0.092 Mol, 61%). ^1H NMR (CDCl_3): δ 6.23ppm (s, 1H, phenol), δ 6.62ppm (s, 1H, phenyl), δ 7.52ppm (s, 1H, phenyl), δ 9.69ppm (s, 1H, formyl), δ 11.25ppm (s, 1H, phenol).

5 To prepare 3-carboxy 6-chloro 7-hydroxy coumarin, 5.76 g (0.033 Mol) 2,4-dihydroxy-5-chlorobenzaldehyde and 7.2 g (0.069 Mol) malonic acid were dissolved in 5 ml warm pyridine. 75 μl Aniline were stirred into the solution and the reaction let to stand at room temperature for 3 days. The
10 yellow solid that formed was broken into smaller pieces and 50 ml ethanol was added. The creamy suspension was filtered through a glass frit and the solid was washed three times with 1 N hydrochloric acid and then with water. Then the solid was stirred with 100 ml ethyl acetate, 150 ml ethanol and 10 ml
15 half concentrated hydrochloric acid. The solvent volume was reduced in vacuo and the precipitate recovered by filtration, washed with diethyl ether and dried over phosphorous pentoxide. 4.97 g (0.021 Mol, 63%) of product was obtained as a white powder. ^1H NMR ($d\text{DMSO}$): δ 6.95ppm (s, 1H), δ 8.02ppm
20 (s, 1H), δ 8.67ppm (s, 1H).

To prepare 7-butyryloxy-3-carboxy-6-chlorocoumarin, 3.1 g (12.9 mMol) 3-carboxy-6-chloro-7-hydroxycoumarin were dissolved in 100 ml dioxane and treated with 5 ml butyric anhydride, 8 ml pyridine and 20 mg dimethyl aminopyridine at
25 room temperature for two hours. The reaction solution was added with stirring to 300 ml heptane upon which a white precipitate formed. It was recovered by filtration and dissolved in 150 ml ethyl acetate. Undissolved material was removed by filtration and the filtrate extracted twice with 50
30 ml 1 N hydrochloric acid/brine (1:1) and then brine. The solution was dried over anhydrous sodium sulfate. Evaporation in vacuo yielded 2.63 g (8.47 mMol, 66%) of product. ^1H NMR (CDCl_3): δ 1.08ppm (t, 3H, $J = 7.4$ Hz, butyric methyl), δ 1.85ppm (m, 2H, $J_1 \sim J_2 = 7.4$ Hz, butyric methylene), δ 2.68ppm
35 (t, 2H, $J = 7.4$ Hz, butyric methylene), δ 7.37ppm (s, 1H, coumarin), δ 7.84ppm (s, 1H, coumarin), δ 8.86ppm (s, 1H, coumarin).

Preparation of 7-butyryloxy-3-

benzyloxycarbonylmethylaminocarbonyl-6-chlorocoumarin is effected as follows. 2.5 g (8.06 mMol) 7-Butyryloxy-3-carboxy-6-chlorocoumarin, 2.36 g hydroxybenztriazole hydrate (16 mMol) and 1.67 g (8.1 mMol) dicyclohexyl carbodiimide were dissolved in 30 ml dioxane. A toluene solution of O-benzylglycine [prepared by extraction of 3.4 g (10 mMol) benzylglycine tosyl salt with ethyl acetate - toluene - saturated aqueous bicarbonate - water (1 : 1 : 1 : 1, 250 ml), drying of the organic phase with anhydrous sodium sulfate and reduction of the solvent volume to 5 ml] was added dropwise to the coumarin solution. The reaction was kept at room temperature for 20 hours after which the precipitate was removed by filtration and washed extensively with ethylacetate and acetone. The combined solvent fractions were reduced to 50 ml on the rotatory evaporator upon which one volume of toluene was added and the volume further reduced to 30 ml. The precipitating product was recovered by filtration and dissolved in 200 ml chloroform - absolute ethanol (1 : 1). The solution was reduced to 50 ml on the rotatory evaporator and the product filtered off and dried in vacuo yielding 1.29 g of the title product. Further reduction of the solvent volume yielded a second crop (0.64 g). Total yield: 1.93g (4.22 mMol, 52%). ¹H NMR (CDCl₃): δ 1.08ppm (t, 3H, J = 7.4 Hz, butyric methyl), δ 1.84ppm (m, 2H, J₁ = J₂ = 7.4 Hz, butyric methylene), δ 2.66ppm (t, 2H, J = 7.4 Hz, butyric methylene), δ 4.29ppm (d, 2H, J = 5.5 Hz, glycine methylene), δ 5.24ppm (s, 2H, benzyl), δ 7.36ppm (s, 1H, coumarin), δ 7.38ppm (s, 5H, phenyl), δ 7.77ppm (s, 1H, coumarin), δ 8.83ppm (s, 1H, coumarin), δ 9.15ppm (t, 1H, J = 5.5 Hz, amide).

7-Butyryloxy-3-carboxymethylaminocarbonyl-6-chlorocoumarin was prepared as follows. 920 mg (2 mMol) 7-butyryloxy-3-benzyloxycarbonylmethylaminocarbonyl-6-chlorocoumarin were dissolved in 50 ml dioxane. 100 mg palladium on carbon (10%) and 100 μl acetic acid were added to the solution and the suspension stirred vigorously in a hydrogen atmosphere at ambient pressure. After the uptake of hydrogen seized the suspension was filtered. The product

containing carbon was extracted five times with 25 ml boiling dioxane. The combined dioxane solutions were let to cool upon which the product precipitated as a white powder. Reduction of the solvent to 20 ml precipitates more product. The
5 remaining dioxane solution is heated to boiling and heptane is added until the solution becomes cloudy. The weights of the dried powders were 245 mg, 389 mg and 58 mg, totaling 692 mg (1.88 mMol, 94%) of white product. ^1H NMR (dDMSO): δ 1.02ppm (t, 3H, $J = 7.4$ Hz, butyric methyl), δ 1.73ppm (m, 2H, $J_1 = J_2 =$
10 7.3 Hz, butyric methylene), δ 2.70ppm (t, 2H, $J = 7.2$ Hz, butyric methylene), δ 4.07ppm (d, 2H, $J = 5.6$ Hz, glycine methylene), δ 7.67ppm (s, 1H, coumarin), δ 8.35ppm (s, 1H, coumarin), δ 8.90ppm (s, 1H, coumarin), δ 9.00ppm (t, 1H, $J =$
15 5.6 Hz, amide).

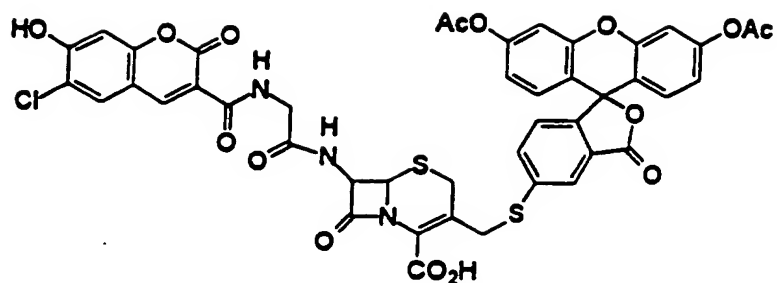
Coupling of 7-Butyryloxy-3-carboxymethylaminocarbonyl-6-chlorocoumarin with 7-amino-3'-chlorocephalosporanic acid benzhydryl ester was effected as follows. 368 mg (1 mMol) 7-Butyryloxy-3-carboxymethylaminocarbonyl-6-chlorocoumarin, 270mg
20 hydroxybenztriazole hydrate and 415 mg (1 mMol) 7-amino-3'-chloro cephalosporanic acid benzhydryl ester were suspended in 40 ml dioxane - acetonitrile (1 : 1). 260 mg (1.25 mMol) dicyclohexylcarbodiimide in 5 ml acetonitrile were added and the suspension was stirred vigorously for 36 hours. The
25 precipitate was removed by filtration and the volume of the solution reduced to 20 ml on the rotatory evaporator. 50ml Toluene was added and the volume reduced to 30 ml. With stirring 50 ml heptane was added and the suspension chilled on ice. The precipitate was recovered by filtration. It was
30 redissolved in 10 ml chloroform and the remaining undissolved solids were filtered off. Addition of 2 volumes of heptane precipitated the title product which was collected and dried in vacuo and yielded 468 mg (0.64 mMol, 64%) off-white powder. ^1H NMR (CDCl_3): δ 1.08ppm (t, 3H, $J = 7.4$ Hz, butyric methyl),
35 δ 1.84ppm (m, 2H, $J_1 = J_2 = 7.4$ Hz, butyric methylene), δ 2.66ppm (t, 2H, $J = 7.4$ Hz, butyric methylene), δ 3.54ppm (2d, 2H, $J = 18.3$ Hz, cephalosporin C-2), δ 4.24ppm (2d, 2H, $J =$
5.8 Hz, cephalosporin 3 methylene), δ 4.37ppm (d, 2H, $J = 3.8$

Hz, glycine methylene), δ 5.02ppm (d, 1H, J = 4.9 Hz, cephalosporin C-6), δ 5.89ppm (dd, 1H, J_1 = 9.0 Hz, J_2 = 5.0 Hz, cephalosporin C-7), δ 6.96ppm (s, 1H, benzhydryl), δ 7.30-7.45ppm (m, 12H, phenyl, coumarin, amide), δ 7.79ppm (s, 1H, coumarin), δ 8.84ppm (s, 1H, coumarin), δ 9.28ppm (t, 1H, J = 3.7 Hz, amide).

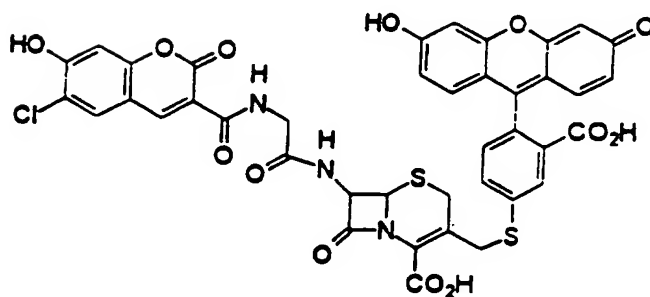
Coupling of the above product with 5-fluoresceinthiol was effected as follows. 90 mg (0.2 mMol) 5-mercaptofluorescein diacetate disulfide dimer were dissolved in 10ml chloroform and treated with 25 μ l tributyl phosphine and 25 μ l water in an argon atmosphere. The solution was kept for 2 hours at ambient temperature and was then added to a solution of 20 mg sodium bicarbonate, 25mg sodium iodide and 110 mg (0.15 mMol) of the above compound in 10 ml dimethylformamide. After 4 hours the solvents were removed in vacuo and the residue triturated with diethylether. The solid was dissolved in ethyl acetate - acetonitrile (1:1). After removal of the solvents the residue was triturated once more with diethylether yielding 157 mg (0.13mMol, 88%) of a cream colored powder product.

A sample of the above compound was treated with a large excess of trifluoroacetic acid - anisole (1:1) at room temperature for 20 minutes. The reagents are removed in vacuo and the residue triturated with ether. High performance liquid chromatography of the solid in 45% aqueous acetonitrile containing 0.5% acetic acid gives a product in which the butyrate and the diphenylmethyl esters have been cleaved. It was purified by high performance liquid chromatography on a reverse phase C_{18} -column using 45% aqueous acetonitrile containing 5% acetic acid as the eluent.

62



27

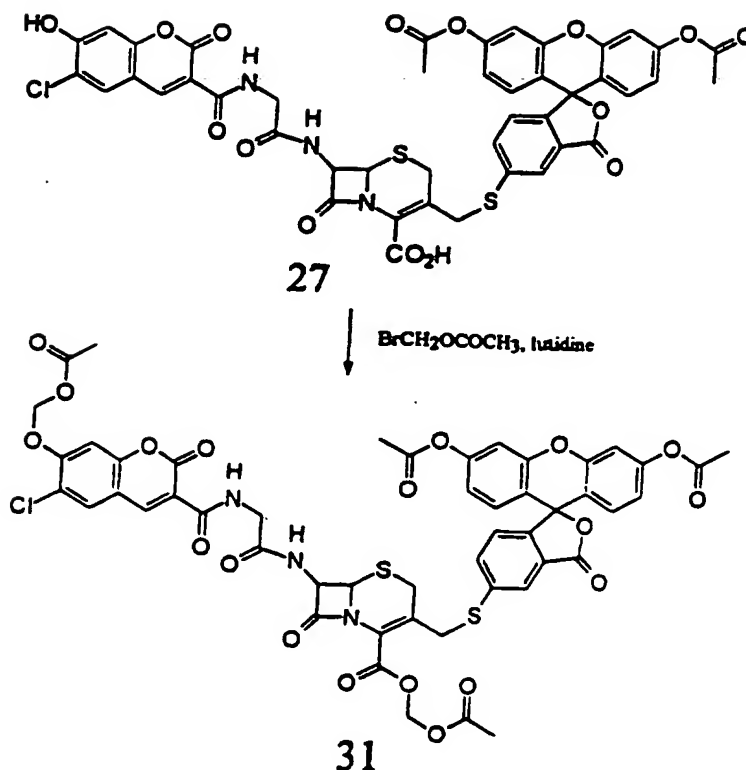
 \downarrow $\text{NaHCO}_3, \text{H}_2\text{O} - \text{CH}_3\text{OH}$ 

7b

CCF2

Deprotection of the fluorescein acetates in compound 27 was accomplished with sodium bicarbonate in methanol (room temperature, 30minutes) to provide the fluorescent enzyme substrate CCF2. It was purified by high performance liquid chromatography on a reverse phase C_{18} - column using 35% aqueous acetonitrile containing 0.5% acetic acid as the eluent.

63



Stirring of compound 27 with excess acetoxymethyl bromide in dry lutidine produced the membrane permeable derivative of the substrate (CCF2/ac₂AM₂). It was purified by high performance liquid chromatography on a reverse phase C₁₈ - column using 65% aqueous acetonitrile containing 0.5% acetic acid as the eluent. CCF2/ac₂AM₂ is readily converted to CCF2 in the cells' cytoplasm.

Unlike in Examples 1-4, the donor and acceptor dyes in substrate CCF2 do not stack. The substrate is fully fluorescent in phosphate buffer and there is no formation of the "dark complex" (i.e., addition of methanol does not change the fluorescence spectrum of CCF2, except for the effect of dilution). This is due to the much smaller and more polar nature of the 7-hydroxycoumarin compared to that of the xanthene dyes (eosin, rhodamine, rhodol and resorufin) in Examples 1-4.

Fig. 5 illustrates the emission spectrum of compound CCF2 in 50 mmolar phosphate buffer pH 7.0 before and after β -lactamase cleavage of the β -lactam ring. In the intact substrate, efficient energy transfer occurs from the 7-

hydroxycoumarin moiety to the fluorescein moiety. Excitation of the substrate at 405 nm results in fluorescence emission at 515 nm (green) from the acceptor dye fluorescein. The energy transfer is disrupted when β -lactamase cleaves the β -lactam ring, thereby severing the link between the two dyes. 5
Excitation of the products at 405 nm now results entirely in donor fluorescence emission at 448 nm (blue). The fluorescence emission from the donor moiety increases 25 fold upon β -lactam cleavage. The fluorescence at 515 nm is reduced 10 by 3.5 fold, all of the remaining fluorescence originating from the 7-hydroxycoumarin as its emission spectrum extends into the green. Twenty-five-fold quenching of the donor in the substrate is equivalent to an efficiency of fluorescence energy transfer of 96%. This large fluorescence change upon 15 β -lactam cleavage can readily be used to detect β -lactamase in the cytoplasm of living mammalian cells, as is reported in Examples 6 and 7.

The 7-hydroxycoumarin moiety in the cephalosporin was determined to have a fluorescence quantum efficiency in 20 the absence of the acceptor of 98-100%. This value was determined by comparing the integral of the corrected fluorescence emission spectrum of the dye with that of a solution of 9-aminoacridine hydrochloride in water matched for absorbance at the excitation wavelength. It follows that 7- 25 hydroxycoumarin is an ideal donor dye, as virtually every photon absorbed by the dye undergoes fluorescence energy transfer to the acceptor.

Example 6

30 Cells of the T-cell lymphoma line Jurkat were suspended in an isotonic saline solution (Hank's balanced salt solution) containing approximately 10^{12} β -lactamase enzyme molecules per milliliter (approximately 1.7 nM; Penicillinase 205 TEM R⁺, from Sigma) and 1 mg/ml rhodamine conjugated to 35 dextran (40 kd) as a marker of loading. The suspension was passed through a syringe needle (30 gauge) four times. This causes transient, survivable disruptions of the cells' plasma membrane and allows entry of labeled dextran and β -lactamase.

Cells that had been successfully permeabilized contained β -lactamase and were red fluorescent when illuminated at the rhodamine excitation wavelength on a fluorescent microscope. The cells were incubated with 5 μ M fluorogenic β -lactamase substrate, CCF2/ac₂AM₂, at room temperature for 30 minutes. Illumination with violet light (405 nm) revealed blue fluorescent and green fluorescent cells. All cells that had taken up the marker rhodamine-dextran appeared fluorescent blue, while cells devoid the enzyme appeared fluorescent green.

Example 7

Cells from cell lines of various mammalian origin were transiently transfected with a plasmid containing the RTEM β -lactamase gene under the control of a mammalian promotor. The gene encodes cytosolic β -lactamase lacking any signal sequence and is listed as SEQ. ID. 1. 10 to 48 hours after transfection cells were exposed to 5 μ mol CCF2/ac₂AM₂ for 1 to 6 hours. In all cases fluorescent blue cells were detected on examination with a fluorescence microscope. Not a single blue fluorescent cell was ever detected in nontransfected control cells. To quantitate the fluorescence measurements the cells were first viewed through coumarin (450 DF 65) and then fluorescein (515 EFLP) emission filters and pictures were recorded with a charge couple device camera. The average pixel intensities of CCF2 loaded transfected cells (blue) and controls (green) at coumarin and fluorescein wavelength in COS-7 (Table 2) and CHO (Table 3) cells are summarized; values for 4 representative cells for each population are given. Thus, the substrate CCF2 revealed gene expression in single living mammalian cells.

Table 2

COS-7 (origin: SV40 transformed african green monkey kidney cells)

	Table of pixel intensities	coumarin emission filter	fluorescein emission filter
5	Blue cell #1	27	20
	#2	34	23
	#3	31	31
	#4	22	33
10	Green cell #1	4	43
	#2	4	42
	#3	5	20
	#4	3	24

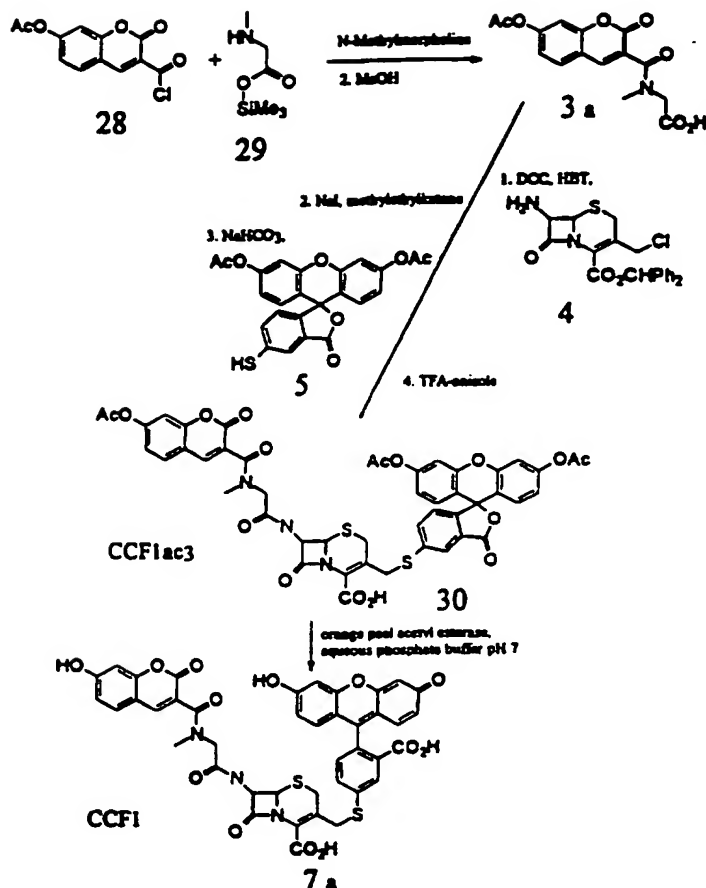
15

Table 3

CHO (origin: Chinese hamster ovary cells)

	Table of pixel intensities	coumarin emission filter	fluorescein emission filter
	Blue cell #1	98	112
20	#2	70	113
	#3	76	92
	#4	56	67
	Green cell #1	9	180
	#2	9	102
25	#3	7	101
	#4	9	83

67

Example 8

For preparation of 7-acetyloxy-3-(N-carboxymethyl-N-methylaminocarbonyl)coumarin, 400 mg (1.6 mMol) 3-carboxy-7-acetylcoumarin were refluxed with 4 ml thionyl chloride for 20 minutes. Excess thionyl chloride was removed by distillation and the residue (7-acetyloxy-3-chlorocarbonylcoumarin) stored in vacuo over potassium hydroxide pellets overnight. In a separate vessel 142.5 mg (1.6 mMol) sarcosine was dissolved in 1.05 ml (5.4 mMol) N-methyl trimethylsilyl trifluoroacetamide (MSTFA) and kept at room temperature for 16 hours. 2 ml dry acetonitrile and 187 μ l (1.7 mMol) N-methylmorpholine were added and the solution was poured onto the solid 7-acetyloxy-3-chlorocarbonylcoumarin on ice. After stirring for 20 minutes on ice the solution was let to warm to room temperature. After 4 hours the solvents were removed in vacuo. The residue was dissolved in methanol to deprotect the acid after which the solvent was removed in vacuo. The solid was dissolved in 30 ml ethylacetate-acetonitrile (2:1) and the solution extracted twice with an equal volume of 1 N hydrochloric acid and the with brine. The organic phase was

dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the solid crystallized from boiling ethylacetate with addition of hexane. The yield was 316mg (1.0 mMol, 63%) of a white crystalline solid.

5 Coupling of 7-acetyloxy-3-(N-carboxymethyl-N-methylaminocarbonyl)coumarin with 7-amino-3'-chlorocephalosporanic acid benzhydryl ester was effected as follows. 62 mg (0.2 mMol) 7-acetyloxy-3-(N-carboxymethyl-N-methylaminocarbonyl)coumarin was stirred with 1 ml dry
10 methylene chloride to which 27 mg (0.2 mMol) hydroxybenztriazole and 41 mg dicyclohexyl carbodiimide had been added. A solution of 82.6 mg (0.2 mMol) 7-amino 3'-chloro cephalosporanic acid benzhydryl ester in 1 ml methylene chloride was added dropwise over a period of 5 minutes. The
15 reaction was stirred for 20 hours at room temperature after which the precipitate was removed by filtration. The filtrate was evaporated in vacuo and the product extracted into methylene chloride. The solvent was removed once more and the residue dissolved in 1 ml ethyl acetate. Addition of three
20 volumes of hexane precipitated the product which was recovered by centrifugation. The yield was 49.9 mg (70 μ Mol, 35%) of the product as a white powder.

 Conversion of the cephalosporin 3'-chloro
substituent in the above product to the 3'-iodo substituent
25 was carried out as follows. 49.9 mg (70 μ Mol) of the above product was stirred with 52.5 mg sodium iodide (5 equivalents) in 1.2 ml dry methyl ethyl ketone at room temperature for 2 hours. The solvent was removed in vacuo and the residue dissolved in 2 ml ethyl acetate - methylene chloride (1:1) and
30 extracted with cold 2% aqueous sodium thiosulfate solution, followed by two extractions with brine. The organic layer was dried over anhydrous sodium sulfate. The slightly orange powder (32 mg, 40 μ Mol, 57%) was used without further purification in the next reaction.

35 Coupling of above product with 5-mercaptofluorescein diacetate (product CCFIac, diphenylmethyl ester) was effected by dissolving 32 mg (40 μ Mol) of the iodo derivative in 0.4 ml dimethylformamide and 3.4 mg sodium bicarbonate added. 22 mg

(50 μ Mol) 5-mercaptofluorescein diacetate were dissolved in 0.3 ml deoxygenated dimethyl formamide and added to the iodo compound in an argon atmosphere. After 2 hours the solvent was removed in vacuo. The residue was suspended in methylene chloride - ethyl acetate (1:1). The organic solution was washed with water and dried over anhydrous sodium sulfate. The solvent was removed and the residue was triturated with ethyl ether hexane (1:1). Flash chromatography on 60 mesh silica gel with ethyl acetate - toluene (2:1) yielded 4.2 mg (4 μ Mol, 10%) of colorless product.

Cleavage of the diphenylmethyl ester to give CCF1ac, was effected as follows. 4 mg (4 μ Mol) of CCF1ac, diphenylmethyl ester were treated with 200 μ l trifluoroacetic acid - anisole - methylene chloride (10:1:10) on ice for 15 minutes. The reagents were removed in vacuo and the residue was dissolved in 0.5 ml ethyl acetate and the solvent evaporated in vacuo. The solid was triturated with ether and then dissolved in 0.5 ml methanol. Addition of the methanolic solution to 2 ml water precipitated the product. The product was recovered by centrifugation and dried in vacuo. The yield was 2mg (2 μ Mol, 50%) white solid. The compound was further purified by high performance liquid chromatography on a reverse phase C_{18} -column using 55% aqueous acetonitrile containing 0.5% acetic acid as the eluent.

The fluorescence emission spectrum of CCF1 before and after β -lactamase cleavage (Fig. 6) was obtained from a sample of CCF1ac, that had been converted to CCF1 by treatment with orange peel acetyl esterase in 50 mmolar aqueous phosphate buffer pH 7.

Substrate CCF1 has similar fluorescence properties to substrate CCF2 in Example 5. In the intact substrate, efficient energy transfer occurs from the 7-hydroxycoumarin moiety to the fluorescein moiety. Excitation of the substrate at 390 nm results in fluorescence emission at 515 nm (green) from the acceptor dye fluorescein. The energy transfer is disrupted when β -lactamase cleaves the β -lactam ring, thereby severing the link between the two dyes. Excitation of the products at 390 nm now results entirely in donor fluorescence

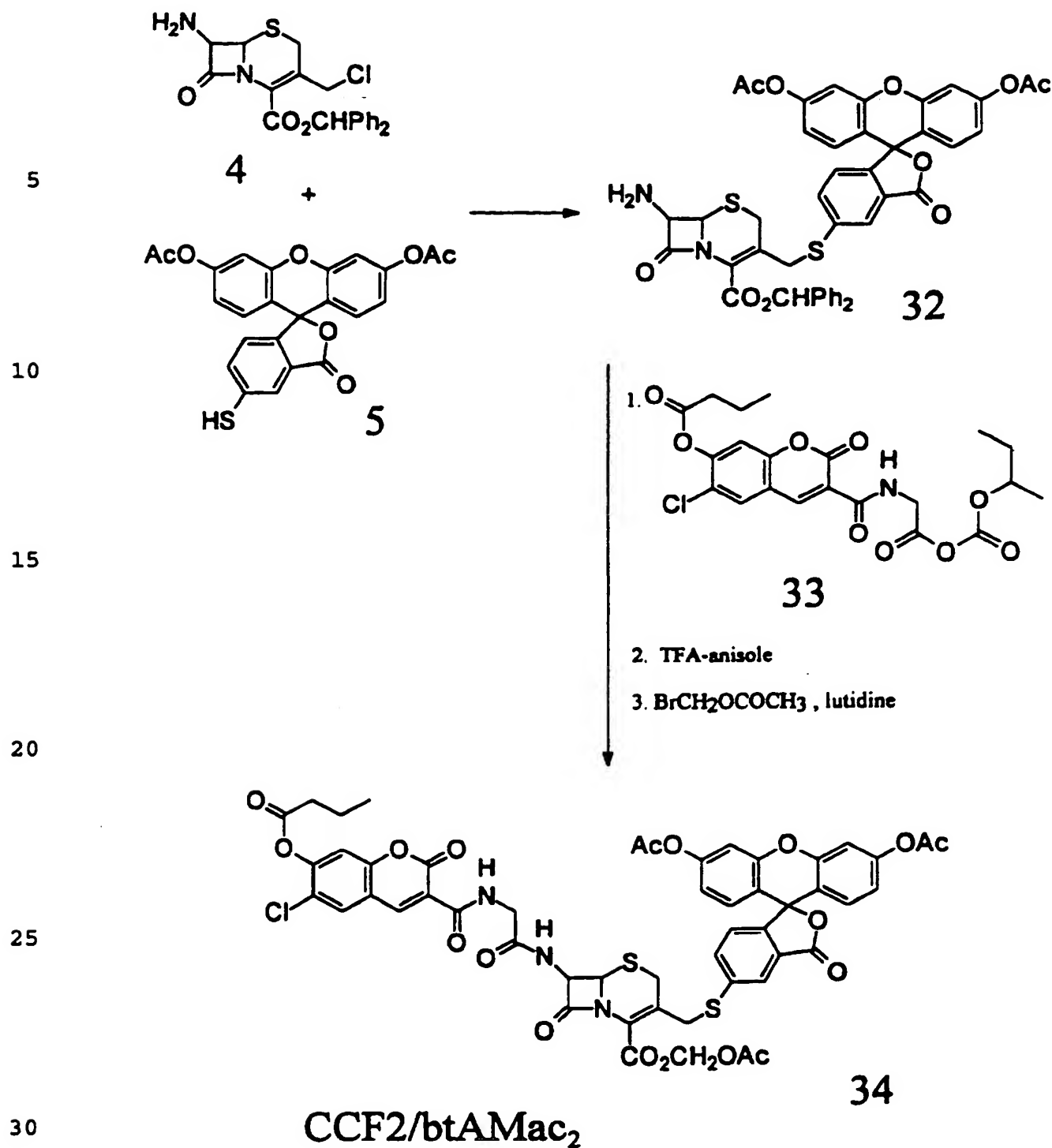
emission at 460 nm (blue). The fluorescence emission from the donor moiety increases 25-fold upon β -lactam cleavage. The fluorescence at 515 nm is reduced by 3-fold, all of the remaining fluorescence originating from the 7-hydroxycoumarin as its emission spectrum extends into the green. Twenty-five-fold quenching of the donor in the substrate is equivalent to an efficiency of fluorescence energy transfer of 96%. This large fluorescence change upon β -lactam cleavage can readily be used to detect β -lactamase in the cytoplasm of living mammalian cells, as is reported in Example 9.

Example 9

Cells of the T-cell lymphoma line Jurkat were suspended in an isotonic saline solution (Hank's balanced salt solution) containing approximately 10^{12} β -lactamase enzyme molecules per milliliter (approximately 1.7 nM; Penicillinase 205 TEM R*, from Sigma) and 1 mg/ml rhodamine conjugated to dextran (40 kd) as a marker of loading. The suspension was passed through a syringe needle (30 gauge) four times. This causes transient, survivable disruptions of the cells' plasma membrane and allows entry of labeled dextran and β -lactamase. Cells which had been successfully permeabilized contained β -lactamase and were red fluorescent when illuminated at the rhodamine excitation wavelength on a fluorescent microscope. The cells were incubated with 30 μ M fluorogenic β -lactamase substrate CCFlac, at room temperature for 30 minutes. Illumination with ultraviolet light (360 nm) revealed blue fluorescent and green fluorescent cells. All cells that had taken up the marker rhodamine-dextran appeared fluorescent blue, while cells devoid the enzyme appeared fluorescent green.

Example 10:

The preferred membrane-permeable ester of CCF2 was prepared as follows:



Coupling of 5-fluoresceinthiol diacetate (5) and 7-amino-3'-chlorocephalosporanic acid benzhydryl ester was effected as follows. 450 mg (1mmol) 5-mercaptofluorescein diacetate disulfide dimer were dissolved in 30ml chloroform and treated with 50 μ l water and 125 μ l tributylphosphine in a nitrogen atmosphere which generated the free 5-fluoresceinthiol. 450 mg (1mmol) 7-Amino-3'-

chlorocephalosporanic acid benzhydryl ester hydrochloride salt were dissolved in 10 ml acetonitrile with the help of 220 μ l (2 mmol) N-methyl morpholine and the two solutions combined after 30 minutes. One hour later the solvent volume was reduced to 5 ml and 50 ml carbon tetrachloride were added. The solvent volume was reduced to 15 ml and hexane added with stirring. The initial orange precipitate consisting mainly of N-methyl morpholine hydrochloride was removed by filtration. Upon further addition of two volumes of hexane 630 mg (0.76 mmol, 76%) white product was precipitated and collected.

The above product was coupled with 7-butyryloxy-3-carboxymethyl aminocarbonyl-6-chlorocoumarin. 325 mg (0.88 mmol) 7-Butyryloxy-3-carboxymethyl aminocarbonyl-6-chlorocoumarin was dissolved in 15 ml hot dry dioxane. With rapid cooling 110 μ l (1 mmol) N-methyl morpholine in 1 ml dioxane and 115 μ l (0.9 mmol) isobutyl chloroformate in 8 ml methylene chloride were added. The reaction was kept at 0°C for 30 minutes after which 661 mg (0.8 mmol) of the above fluorescein-cephalosporin adduct in 7 ml dry methylene chloride were added. The solution was let to warm to room temperature and after 3 hours the solvents were removed in vacuo. The residue was dissolved in 30 ml methylene chloride and twice extracted with one volume 10% aqueous acetic acid and once with water. The organic phase was dried over anhydrous sodium sulfate. Addition of 150 ml dry ethanol, reduction of the solvent volume to 50 ml and cooling to -20°C resulted in precipitation of the product (crude, 850mg). Purification was achieved by chromatography over silica gel with 25% ethyl acetate in toluene as the eluent. 250 mg (0.21 mmol, 26%) of white powdorous product was collected.

Cleavage of the cephalosporin benzhydryl ester was accomplished by treatment with trifluoroacetic acid. 145 mg (0.12 mmol) of the above product was treated with trifluoroacetic acid / methylene chloride / anisole (10 / 10 / 1) at 0°C for 20 minutes. The reagents were removed in vacuo and the residue triturated with diisopropyl ether. The solid was dissolved in 1 ml dimethyl sulfoxide and the product precipitated by addition to 25 ml water. It was further

purified on reverse phase C₁₈ resin with a step gradient of 40 to 60% aqueous acetonitrile containing 0.5% acetic acid as the eluent yielding 74 mg (73 μ mol, 60%) white powder.

Protection of cephalosporin acid as membrane permeable acetoxymethyl ester was achieved as follows. 15 mg (15 μ mol) of the above product was dissolved in 250 μ l methylene chloride. 25 μ l Bromomethyl acetate and 50 μ l lutidine were added to the solution. The reaction was kept at ambient temperature for 7 hours after which the reagents were removed in vacuo. The residue was purified by flash chromatography on silica gel with ethyl acetate as the eluent. 15 mg (14 μ mol, 92%) white product was obtained. This compound, named CCF2/btAMac₂, was used for intracellular detection of β -lactamase activity.

Example 11:

Measurement of activation of an intracellular receptor: Activation of the intracellular glucocorticoid receptor was measured by its ability to upregulate the transcriptional activity of the glucocorticoid responsive element in the mouse mammary tumor virus promotor. This response to steroids was detected as increased intracellular β -lactamase activity on the substrate CCF2 causing an appropriate change in fluorescent signal.

The gene for plasmid encoded RTEM β -lactamase of *Escherichia coli* without a signal sequence (Sequence 1 of Fig. 7) was put under transcriptional control of the mouse mammary tumor virus promotor and introduced into a mammalian expression vector. This vector also carried the chloramphenicol resistance marker for amplification of the plasmid in bacteria and the neomycin resistance marker for mammalian selection. It was introduced into baby hamster kidney (BHK) cells in culture using the calcium phosphate precipitation technique. Cells were then subjected to selection for stable integration of the plasmid into the cells' genome using the antibiotic G418. One of twenty clones was selected for its marked increase in β -lactamase expression following exposure to the steroid analog dexamethasone.

The following describes the measurement of the increase in β -lactamase gene expression in this clone after addition of the agonist dexamethasone. Cells of the stable BHK cell clone G941 expressing β -lactamase under control of the glucocorticoid-inducible promotor were kept in the presence or absence of the agonists in the incubator at 37 °C. Flasks with cells were removed from the incubator at different intervals after agonist addition and the cells transferred into Hank's balanced salt solution containing 10 μ molar CCF2/btAMac₂. This compound becomes converted to the β -lactamase accessible fluorescent substrate CCF2 by endogenous cytoplasmic esterases. Ten minutes later the cell supernatant containing CCF2/btAMac₂ was removed. 30 Minutes later the cells were imaged with a cooled CCD camera mounted on an epifluorescence microscope. Fluorescence measurements were taken with violet excitation light (filter 400DF15) and with blue (filter 450DF65) and green (filter 535DF45) emission filters. A ratio of blue versus green emission intensities was determined. The ratio is a measure of how much substrate has been converted to product. Using a 40x objective, 4 fields with approximately 60 cells each were imaged at each time point. The results show a significant increase in the ratio of fluorescent intensities reflective of increasing β -lactamase expression and production.

time in presence of 1 μ M dexamethasone	0.0 hours	1.0 hours	2.0 hours	3.3 hours
average ratio of fluorescence intensities 450DF65 / 535DF45	0.21 +/- 0.02	0.38 +/- 0.05	0.42 +/- 0.07	0.47 +/- 0.08

Example 12:

Measurement of cell surface receptor activation and intracellular signaling via second-messenger responsive

elements: Activation of cell surface receptors leads to a change in intracellular messenger concentrations which in turn modulates intracellular transcription factor activity. In lymphocytes, an increase the intracellular concentration of the messenger ion calcium leads to the activation of the nuclear factor of activated T-lymphocytes (NFAT). This event increases transcription at promoters containing the NFAT-recognition site. An increase in calcium levels alone is sufficient to markedly increase transcription of a reporter gene such as β -lactamase regulated when it is put under transcriptional control of a promotor containing a trimer of NFAT sites.

The murine T-lymphocyte cell line B3Z was transiently cotransfected with two plasmids. One plasmid contained the β -adrenergic receptor, which localizes at the cells' surface, under the transcriptional control of the strong and constitutively active cytomegalovirus (CMV) promoter. The other plasmid contained the bacterial RTEM β -lactamase gene from *Escherichia coli* modified for improved mammalian expression (sequence ID # 3 , with optimum mammalian Kozak sequence, β -globin leader sequence, pre-sequence removed) under the transcriptional control of a promotor containing a trimer of NFAT sites. The plasmids were introduced into cells using electroporation. 5×10^6 cells in 0.5 ml electroporation buffer were electroporated in the presence of 10 μ g each of both plasmids using the Biorad Gene Pulser (250V, 960 μ F, 16 μ sec). Twenty-four hours after transfection, cells were either incubated in the presence or absence of the β -adrenergic agonist isoproterenol (10 μ molar) for 5 hours. The supernatant was removed and replaced with Hank's balanced salt solution containing 10 μ molar CCF2/btAMac₂. After 20 minutes at room temperature cells were washed with fresh buffer and viewed with the fluorescence microscope. 4% of isoproterenol treated cells appeared fluorescent blue (excitation filter 400DF15, emission filter 435 nm longpass) while no blue fluorescent cells were detectable in the control population (absence of agonist). Maximal stimulation with 2 μ M ionomycin and 50 ng/ml phorbol

ester for 5 hours resulted in 20% blue fluorescent cells in the population.

Example 13:

5 β -Lactamases from different microorganisms were modified for use as reporter enzymes in eukaryotic cells, preferably mammalian. The bacterial gene for these enzymes includes a N-terminal pre-sequence (first 23 amino acids of Sequence 2 of Fig. 7.) that targets the enzyme to the
10 extracellular space. Following translocation a pre-sequence peptidase cleaves the 23 amino acid pre-sequence releasing the mature β -lactamase enzyme. RTEM β -lactamase from *Escherichia coli* including its bacterial pre-sequence (Sequence 2 of Fig. 7) was put into a mammalian expression vector under the
15 control of the mouse mammary tumor virus promotor. This construct was introduced into baby hamster kidney cells using the standard calcium phosphate precipitation technique. The β -lactamase activity was found in the cell culture medium; no activity could be detected in the cell pellet. The amount of
20 β -lactamase activity in the medium was steroid dependent. Cells that had been in the presence of 1 μ M dexamethasone for 36 hours prior to the measurement produced threefold more enzyme than control. This makes the β -lactamase with its bacterial pre-sequence (Sequences 2 of Fig. 7) useful for an
25 extracellular assay of mammalian reporter gene activity.

A preferred use of the β -lactamase reporter is where the enzyme is produced and retained in the cell cytoplasm. Therefore the bacterial signal sequence was removed and replaced by ATG (methionine) as the new translational start
30 site in three modified RTEM β -lactamase genes (Sequences 1, 3, and 4 of Fig. 7). In order to increase expression of the β -lactamases in mammalian cells, the RTEM β -lactamases of Sequence 3 and 4 of Fig. 7 were constructed with altered ribosome binding sites optimized for mammalian expression
35 [Kozak, M., *J. Cell Biol.* 108: 229-241 (1989)]. For increased compatibility with the mammalian translation machinery, β -lactamase of sequence ID #3 was inserted at the end of an untranslated mammalian β -globin leader sequence. All of

these novel DNA sequences encoding novel β -lactamases were inserted into mammalian expression vectors with the cytomegalovirus promotor controlling their transcription. Mammalian cells in tissue culture (Hela, COS-7, CHO, BHK) were transfected transiently with the plasmids using the standard lipofectin technique. Two to five days after transfection, the cells were incubated with the membrane-permeant derivative, CCF2/btAMac₂, of the fluorescent substrate CCF2 to assay functional expression of the enzyme. 5-20% of cells transfected with plasmids containing cDNA Sequences 2, 3 and 4 of Fig. 7 showed a conversion of green to blue fluorescence indicating cleavage of the intracellularly trapped substrate by expressed β -lactamase. By contrast, in untransfected or mock transfected controls, all cells showed the green fluorescence of uncleaved CCF2; no blue-fluorescing cells were observed, confirming the absence of any endogenous β -lactamase activity.

The gene for *Bacillus licheniformis* β -lactamase was isolated from total *Bacillus licheniformis* DNA by use of the polymerase chain reaction. The oligonucleotide primers removed the β -lactamase secretion sequence and generated the DNA sequence ID # 5. This gene was inserted in a pCDNA3 mammalian expression vector under the transcriptional control of the constitutively active cytomegalovirus promoter. HeLa cells were transfected with 10 μ g of plasmid per 25 cm² culture dish using lipofectin. 5 days after transfection, cells were tested for functional expression of β -lactamase by incubating them in the presence of 100 μ molar CCF2/btAMac₂ and visual inspection with the epifluorescence microscope. 30-40% of cells showed blue fluorescence, whereas only green-fluorescing cells, no blue-fluorescing cells were detectable in untransfected controls. In transient transfections, it is typical for <50% of the cells to become transfected.

Example 14

A plasmid was constructed with β -lactamase of sequence ID 3 (figure 7) under control of yeast elongation factor EF-1 α enhancer and promoter. This plasmid was

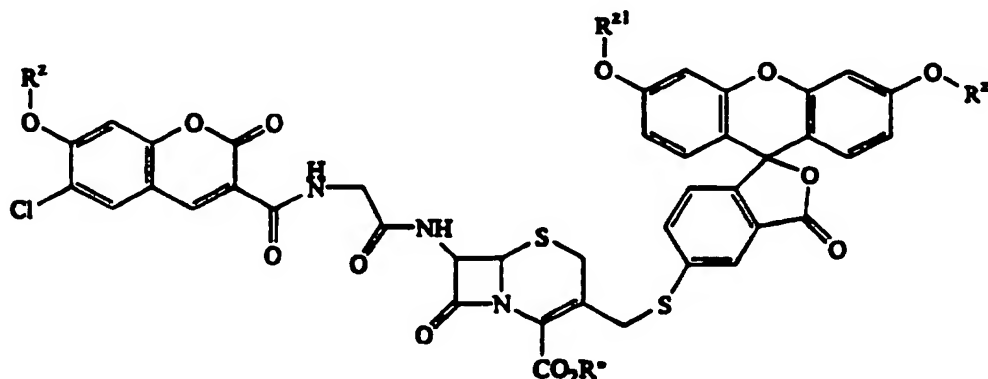
coinjecting together with the potassium salt of substrate CCF2 (compound 7b) into zebrafish embryos at the single cell stage. As control, embryos were injected with the potassium salt of substrate CCF2 alone. After three hours, the embryos were
5 viewed with an epifluorescence microscope using violet excitation light (filter 400DF15) and a 435 nm longpass emission filter. Embryos that had received plasmid DNA fluoresced blue while controls fluoresced green.

10 Example 15

The β -lactamase gene of sequence ID 3 was cloned into a *Drosophila* transformation vector under the control of the glass promoter and injected into wild-type *Drosophila* embryos. As control, the β -lactamase gene was inserted in the
15 wrong orientation. *Drosophila* embryos were germline-transformed using P element-mediated transformation. The transformations and all subsequent fly manipulations were performed using standard techniques [Karess, R.E. and Rubin, G.M., *Cell* 38, 135, (1984)]. Omatidia of late stage
20 transformed pupae were transsected and dissociated to single cells. The cells were incubated in buffer with 40 μ molar CCF2/btAMac₂ (compound 34) for 20 minutes, washed and viewed with an epifluorescence microscope (excitation filter 400DF15, emission filter 435 nm long pass). Omatidia cells from flies
25 transformed with the β -lactamase gene in the proper orientation fluoresced blue, while omatidia cells containing the gene in the wrong orientation fluoresced green.

Example 16

30 In certain embodiments the compound of this invention can be any of the following compounds.



wherein

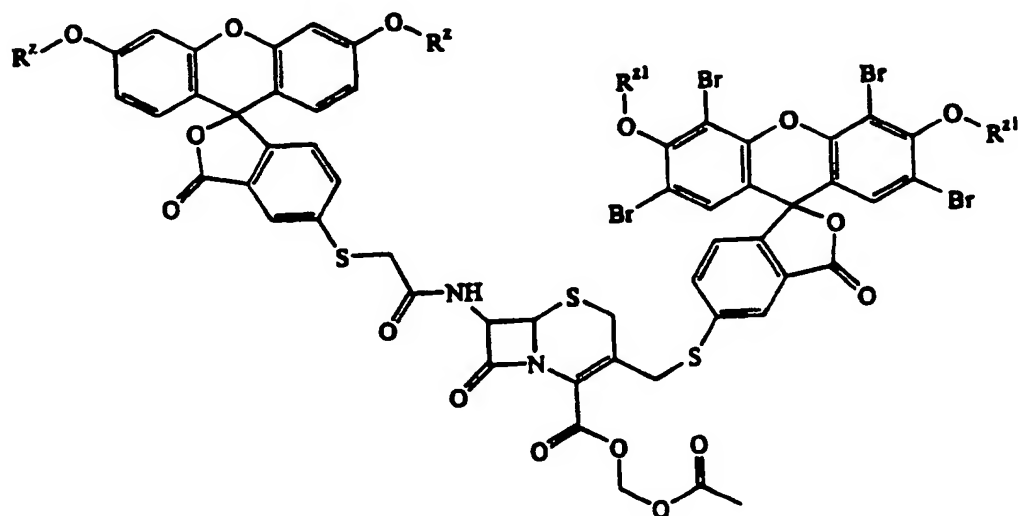
R^Y is selected from the group consisting of H, Cl, and Br;

R^X is selected from the group consisting of H, and methyl;

wherein R^Z and R^{Z1} are independently selected from the group consisting of $-C(O)alk$, $-CH_2OC(O)alk$, $-CH_2SC(O)alk$, $-CH_2OC(O)Oalk$, lower acyloxy-alpha-benzyl, and deltabutyrolactonyl; wherein alk is lower alkyl of 1 to 4 carbon atoms, and membrane-permeant fluorogenic derivatives thereof,

R^* is 1-(acyloxy)alkyl.

Another example of the compound is:



wherein R^Z and R^{Z1} are independently selected from the group consisting of $-C(O)alk$, $-CH_2OC(O)alk$, $-CH_2SC(O)alk$, $-CH_2OC(O)alk$, lower acyloxy-alpha-benzyl, and deltabutyrolactonyl; wherein alk is lower alkyl of 1 to 4 carbon atoms.

All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

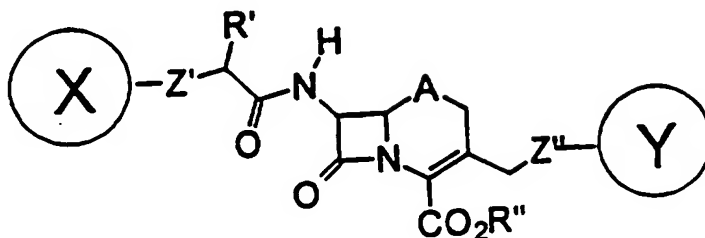
5 The present invention provides novel substrates for beta-lactamase, beta-lactamases and methods for their use. While specific examples have been provided, the above description is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

10

15 All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

WHAT IS CLAIMED IS:

1. A compound of the formula (I):



2 wherein:

3 one of X and Y is a fluorescent donor moiety or a
4 membrane-permeant derivative thereof, and the other is a
5 quencher moiety, an acceptor fluorophore moiety or a membrane-
6 permeant derivative thereof;

7 R' is selected from the group consisting of H, lower
8 alkyl, $(CH_2)_nOH$, $(CH_2)_nCOOR''$, and $=NOJ$, in which n is 0 or an
9 integer from 1 to 5 and J is H, Me, CH_2COOH , $CHMeCOOH$, and
10 CMe_2COOH ;

11 R'' is selected from the group consisting of H,
12 physiologically acceptable metal and ammonium cations, -
13 $CHR^2OCO(CH_2)_nCH_3$, $-CHR^2OCOC(CH_3)_3$, acylthiomethyl, acyloxy-alpha-
14 benzyl, delta-butyrolactonyl, methoxycarbonyloxymethyl,
15 phenyl, methylsulphinylmethyl, betamorpholinoethyl,
16 dialkylaminoethyl, dialkylaminocarbonyloxymethyl, in which R²
17 is selected from the group consisting of H and lower alkyl;

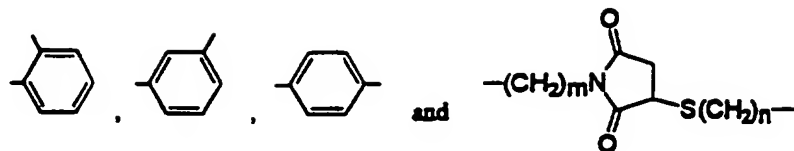
18 A is selected from the group consisting of S, O, SO,
19 SO₂ and CH₂;

20 Z' is a linker for X; and

21 Z'' is a linker for Y.

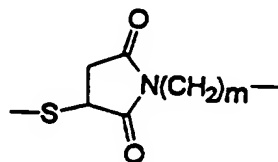
2. The compound of claim 1, wherein Z' is selected
from the group consisting of a direct bond,
- $(CH_2)_nCONR^2(CH_2)_m-$, - $(CH_2)_nNR^2CO(CH_2)_m-$, - $(CH_2)_nNR^3CONR^2(CH_2)_m-$, -
- $(CH_2)_nNR^3CSNR^2(CH_2)_m$, - $(CH_2)_nCONR^3(CH_2)_pCONR^2(CH_2)_m-$, - $(CH_2)_n-$, -
- $(CH_2)_nNR^3CO(CH_2)_pS(CH_2)_m-$, - $(CH_2)_nS(CH_2)_m-$, - $(CH_2)_nO(CH_2)_m-$,

6 $-(\text{CH}_2)_n\text{NR}^2(\text{CH}_2)_m-$, $-(\text{CH}_2)_n\text{SO}_2\text{NR}^2(\text{CH}_2)_m-$, $-(\text{CH}_2)_n\text{CO}_2(\text{CH}_2)_m-$,



7 wherein R^2 and n are as previously defined; R^3 is selected from
 8 the group consisting of hydrogen and lower alkyl; and each of
 9 m and p is independently selected from the group consisting of
 10 integers from 0 to 4.

1 3. The compound of claim 1 wherein Z'' is selected
 2 from the group consisting of a direct bond to a heteroatom
 3 selected from the group consisting of O, N and S in the
 4 chromophore,
 5 $-\text{O}(\text{CH}_2)_n-$, $-\text{S}(\text{CH}_2)_n-$, $-\text{NR}^2(\text{CH}_2)_n-$, $-\text{N}^+\text{R}_2(\text{CH}_2)_n-$,
 6 $-\text{OCONR}^2(\text{CH}_2)_n-$, $-\text{O}_2\text{C}(\text{CH}_2)_n-$, $-\text{SCSNR}^2(\text{CH}_2)_n-$, $\text{SCSO}(\text{CH}_2)_n-$,
 7 $-\text{S}(\text{CH}_2)_n\text{CONR}^2(\text{CH}_2)_m$, $-\text{S}(\text{CH}_2)_n\text{NR}^2\text{CO}(\text{CH}_2)_m$, and



8 wherein R^2 and n are as previously defined; and m is an
 9 integer from 0 to 4.

1 4. The compound of claim 1 wherein R' is selected
 2 from the group consisting of H and methyl.

1 5. The compound of claim 4 wherein R' is H.

1 6. The compound of claim 1 wherein R' is selected
 2 from the group consisting of H and acetoxymethyl.

1 7. The compound of claim 1 wherein R² is H.

1 8. The compound of claim 1 wherein A is -S-.

1 9. The compound of claim 1 wherein one of X and Y
2 is a membrane-permeant derivative of the fluorescent donor
3 moiety, or a membrane-permeant derivative of the quencher
4 moiety or acceptor fluorophore moiety, and wherein at least
5 one of X and Y contains at least one acylated aromatic
6 hydroxyl, acylated amine, or alkylated aromatic hydroxyl
7 wherein the acyl group contains 1 to 5 carbon atoms and
8 wherein the alkyl group is selected from the group consisting
9 of
10 -CH₂OC(O)alk, -CH₂SC(O)alk, -CH₂OC(O)Oalk, lower acyloxy-alpha-
11 benzyl, and deltabutyrolactonyl; wherein alk is lower alkyl of
12 1 to 4 carbon atoms.

1 10. The compound of claim 9 wherein at least one of
2 X and Y contains at least one acylated aromatic hydroxyl,
3 wherein the acyl group is either acetyl, n-propionyl, or n-
4 butyryl.

1 11. The compound of claim 1 wherein said donor is a
2 coumarin of formulae II-VII and said quencher or acceptor is
3 selected from the group consisting of fluoresceins, rhodols,
4 and rhodamines of formulae VIII-XII, XLVII, and XLVII.

1 12. The compound of claim 11 wherein said donor is
2 selected from the group consisting of coumarins of formulae
3 II-VII and fluoresceins of formula VIII.

1 13. The compound of claim 1 wherein said quencher
2 or acceptor is selected from the group consisting of
3 fluoresceins, rhodols, and rhodamines of formulae VIII-XII.

1 14. The compound of claim 13 wherein said donor is
2 a fluorescein of formula VIII and said quencher or acceptor is

3 selected from the group consisting of rhodols and rhodamines
4 of formulae VIII-XII.

1 15. The compound of claim 12 wherein said coumarin
2 is selected from the group consisting of 7-hydroxycoumarin and
3 7-hydroxy-6-chlorocoumarin and said fluorescein is selected
4 from the group consisting of fluorescein and
5 dichlorofluorescein.

1 16. The compound of claim 13 wherein said donor is
2 a fluorescein and said quencher or acceptor is selected from
3 the group consisting of an eosin or a tetrachlorofluorescein
4 of formula VIII in which R^a , R^b , R^c , and R^d are Br or Cl.
5

1 17. The compound of claim 13 wherein said quencher
2 or acceptor is a rhodol of formulae VIII, IX, or XI.

1 18. The compound of claim 13 wherein said quencher
2 or acceptor is a rhodamine of formulae VIII, X, or XII.

1 19. The compound of claim 9 that is membrane-
2 permeant derivatives wherein at least one of X and Y contains
3 an acetoxymethyl group on an aromatic hydroxyl group.

1 20. The compound of claim 2 wherein Z' is
2 $-(CH_2)_nCONR^2(CH_2)_m-$.

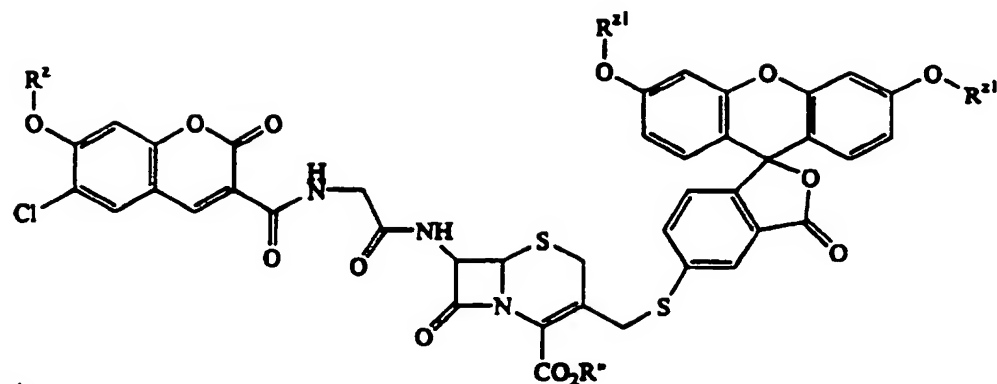
1 21. The compound of claim 20 wherein n and m are 0.

1 22. The compound of claim 20 wherein R^2 is H.

1 23. The compound of claim 3 wherein Z' is $-S(CH_2)_n-$.

1 24. The compound of claim 23 wherein n is 0.

1 25. The compound of claim 12 of the formula:



2 wherein

3 R^y is selected from the group consisting of H, Cl, and
4 Br;

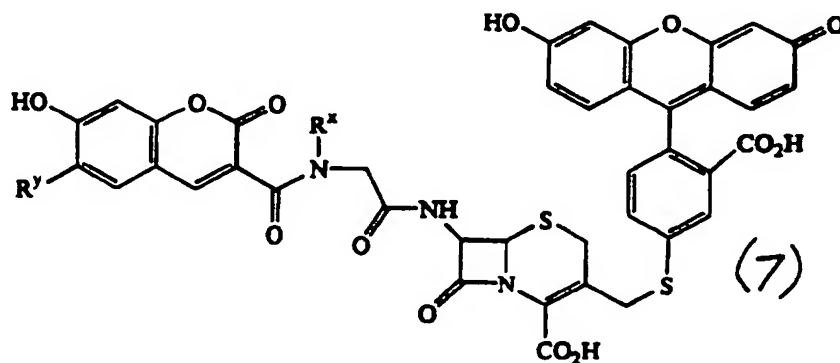
5 R^x is selected from the group consisting of H, and
6 methyl;

7 wherein R^z and R^{z1} are independently selected from the
8 group consisting of -C(O)alk, -CH₂OC(O)alk, -CH₂SC(O)alk, -
9 CH₂OC(O)Oalk, lower acyloxy-alpha-benzyl, and
10 deltabutyrolactonyl; wherein alk is lower alkyl of 1 to 4
11 carbon atoms, and membrane-permeant fluorogenic derivatives
12 thereof,

13 R⁺ is 1-(acyloxy)alkyl

1 26. The compound of claim 25 wherein R^z and R^{z1} are
2 selected from the group consisting of acetyl, butyryl, and
3 acetoxymethyl.

1 27. The compound of claim 12 of the formula:

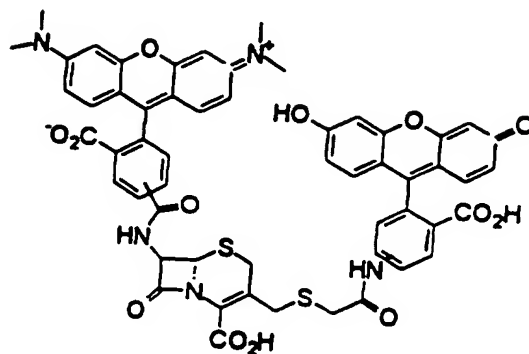


2 wherein

3 R^y is selected from the group consisting of H, Cl, and
4 Br; and

5 R^x is selected from the group consisting of H, and
6 methyl.

1 28. The compound of claim 14 of the formula (11):

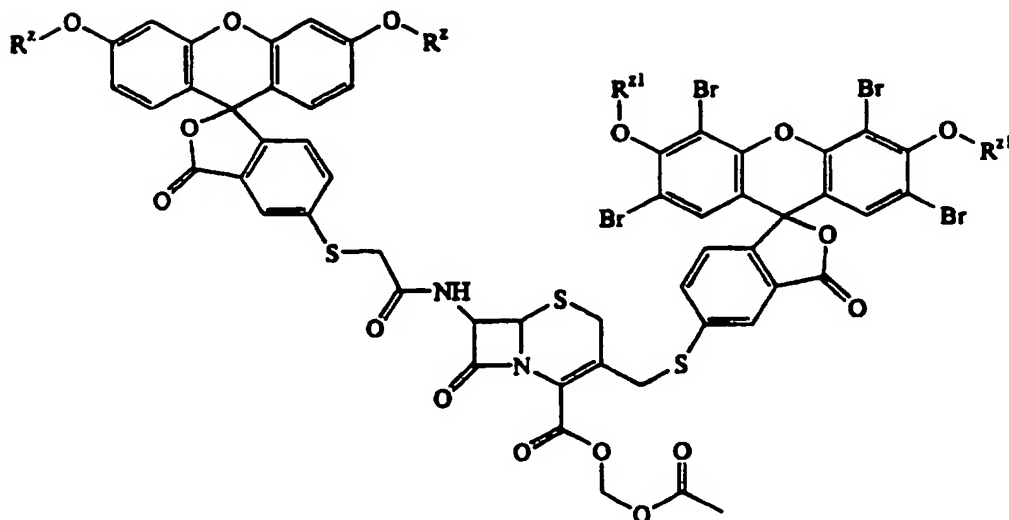


RCF

11

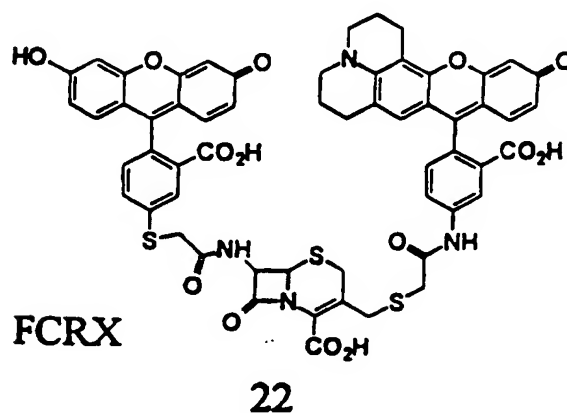
2 and membrane-permeant fluorogenic derivatives thereof.

1 29. The compound of claim 16 of the formula (16):



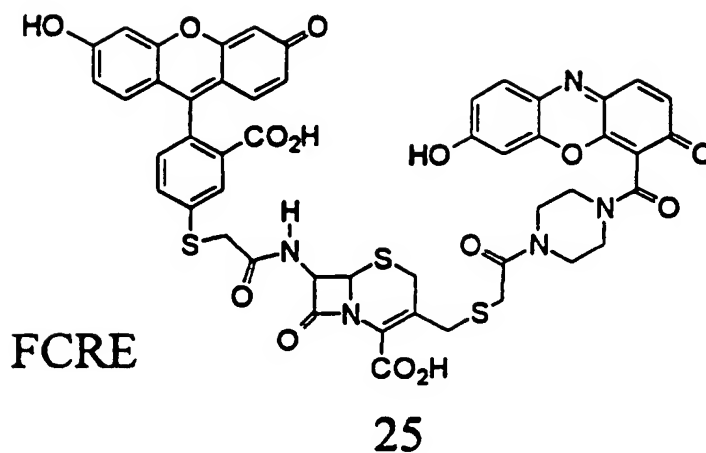
2 wherein R^2 and R^{21} are independently selected from the group
3 consisting of $-C(O)alk$, $-CH_2OC(O)alk$, $-CH_2SC(O)alk$, $-$
4 $CH_2OC(O)alk$, lower acyloxy-alpha-benzyl, and
5 deltabutyrolactonyl; wherein alk is lower alkyl of 1 to 4
6 carbon atoms.

1 30. The compound of claim 14 of the formula (22):



2 and membrane-permeant fluorogenic derivatives thereof.

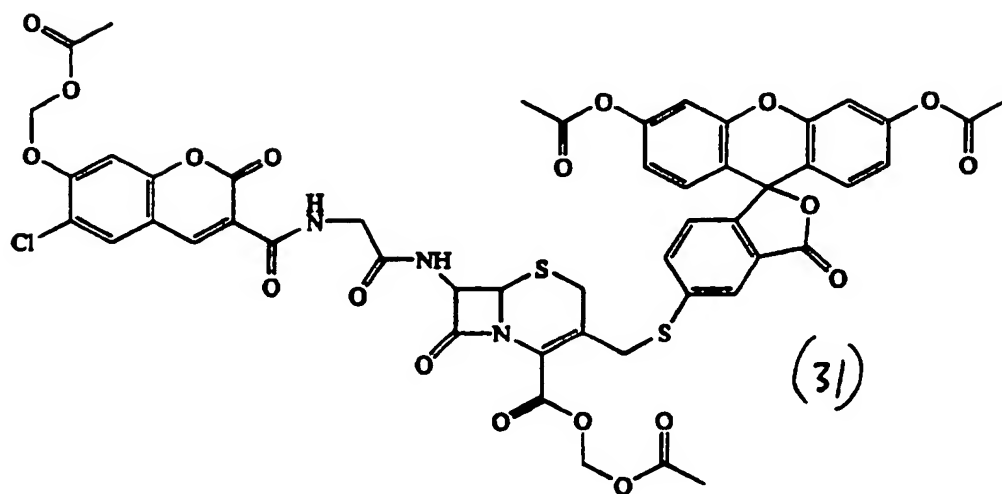
- 1 31. The compound of claim 1 of the formula (25):



- 2 and membrane-permeant fluorogenic derivatives thereof.

- 1 32. The compound of claim 27 wherein R^y is Cl and R^x
2 is H. (CCF2 or 76).

- 1 33. The compound of claim 15 that is



1 34. The compound of claim 26 wherein
2 R^2 and R^1 are acetoxymethyl;
3 R^x is H; and
4 each R^{z1} is acetyl.

1 35. The compound of claim 27 wherein
2 R^y is H; and
3 R^x is methyl (CCF1 7a).

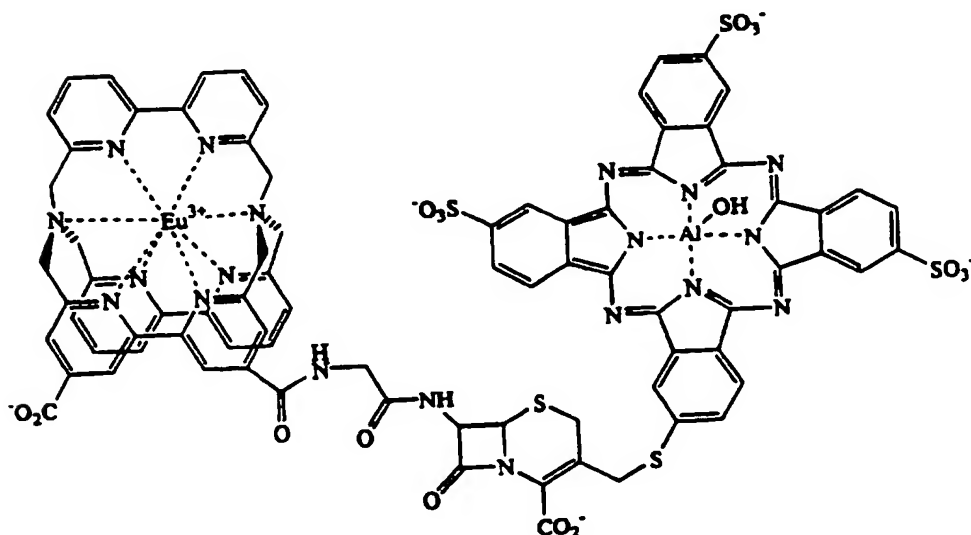
1 36. The compound of claim 1 wherein said donor is
2 selected from the group consisting of fluorescent europium and
3 terbium complexes.

1 37. The compound of claim 36 wherein said donor is
2 selected from the group consisting of europium and terbium
3 tris-(bipyridine) cryptands and related dyes.

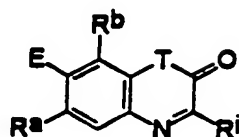
1 38. The compound of claim 1 wherein said quencher
2 or acceptor is selected from the group consisting of
3 phthalocyanines and related dyes.

1 39. The compound of claim 37 wherein said quencher
2 or acceptor is selected from the group consisting of
3 phthalocyanines and related dyes.

1 40. The compound of claim 39 that is



- 1 41. The compound of claim 10 wherein said coumarin
2 is a compound of formula (III):



III

3 wherein

4 E is selected from the group consisting of H, OH, OR^k,
5 and NR^gR^h;

6 T is selected from the group consisting of O and NR^k;

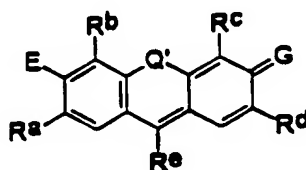
7 R^a and R^b are independently selected from the group
8 consisting of an attachment point, H, halogen, and lower
9 alkyl;

10 R^g, R^h, and R^k are independently selected from the group
11 consisting of an attachment point, H, lower alkyl, and -
12 CH₂(CH₂)_na;

13 Rⁱ is selected from the group consisting of an attachment
14 point, H, halogen, lower alkyl, CN, CF₃, phenyl, CO₂H, and
15 CONR^gR^h; and

16 a is selected from the group consisting of H or an
17 attachment point; and membrane-permeant fluorogenic
18 derivatives thereof.

- 1 42. The compound of claim 11 wherein said acceptor
2 or quencher is a compound of formula (VIII):



VIII

3 wherein

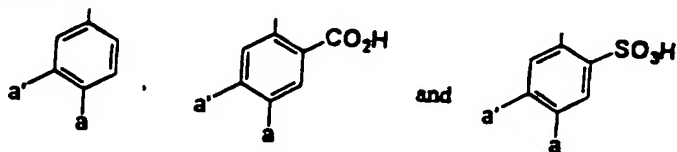
4 E is selected from the group consisting of H, OH, OR^k,
5 and R^gR^h;

6 G is selected from the group consisting of O and N⁺R^gR^h;

7 Q' is selected from the group consisting of O, CH₂,
8 C(CH₃)₂, and NR^g;

R^a , R^b , R^c , and R^d are independently selected from the group consisting of an attachment point, H, halogen, and lower alkyl;

R^e is selected from the group consisting of an attachment point, H, lower alkyl, $(CH_2)_nCO_2H$, $(CH_2)_nCHaCO_2H$, $CHa(CH_2)_nCO_2H$, $(CH_2)_nCOa$, $CH=CHCOa$;



R^g , R^h , R^k are independently selected from the group consisting of an attachment point, H, lower alkyl, and $-CH_2(CH_2)_na$;

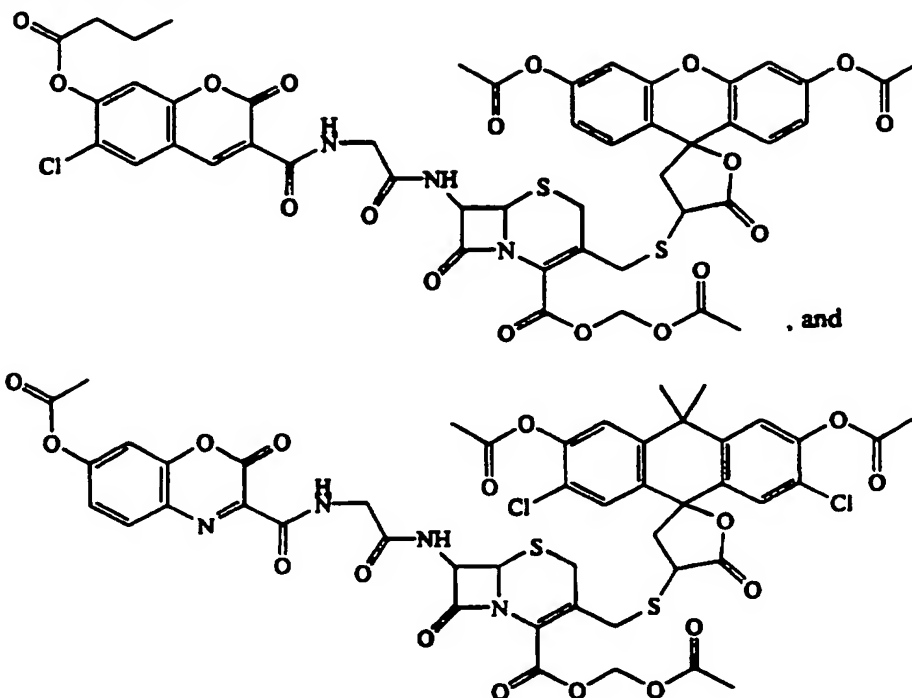
n is an integer of from 0 to 5;

a and a' are independently selected from the group consisting of H or an attachment point; and membrane-permeant fluorogenic derivatives thereof.

43. The compound of claim 42 wherein said donor is selected from the group consisting of coumarins of formulae II-VII, and membrane-permeant fluorogenic derivatives thereof.

44. The compound of claim 43 wherein
 Q' is $C(CMe_2)_2$; and
 G is O; and membrane-permeant fluorogenic derivatives thereof.

45. The compound of claim 43 selected from the group consisting of:



46. A method for determining whether a sample contains β -lactamase activity comprising:

- contacting the sample with a compound of claim 1, which exhibits fluorescence resonance energy transfer when the compound is excited;
- exciting the compound; and
- determining the degree of fluorescence resonance energy transfer in the sample, whereby a degree of fluorescence resonance energy transfer that is lower than an expected amount indicates the presence of β -lactamase activity.

47. The method of claim 1 for determining the amount of an enzyme in a sample wherein determining the degree of fluorescence resonance energy transfer in the sample comprises determining the degree at a first and second time after contacting the sample with the substrate, and determining the difference in the degree of fluorescence resonance energy transfer, whereby the difference in the degree of fluorescence resonance energy transfer reflects the amount of enzyme in the sample.

1 48. A recombinant nucleic acid molecule comprising
2 expression control sequences adapted for function in a
3 vertebrate cell and operably linked to a nucleotide sequence
4 coding for the expression of a β -lactamase.

1 49. The recombinant nucleic acid molecule of claim
2 48 wherein the nucleotide sequence encodes a class A β -
3 lactamase.

1 50. The recombinant nucleic acid molecule of claim
2 49 further comprising mammalian Kozak sequences for the
3 ribosome binding site.

1 51. The recombinant nucleic acid molecule of claim
2 50 wherein the nucleotide sequence is Sequence 3 or Sequence 4
3 of Figure 7.

1 52. The recombinant nucleic acid molecule of claim
2 49 wherein the nucleotide sequence is Sequence 1 or Sequence 5
3 of Figure 7.

1 53. The recombinant nucleic acid molecule of claim
2 48 wherein the expression control sequences are inducible
3 expression control sequences.

1 54. The recombinant nucleic acid molecule of claim
2 48 wherein the expression control sequences are constitutively
3 active expression control sequences.

1 55. The recombinant nucleic acid molecule of claim
2 48 wherein the expression control sequences include the c-fos
3 promoter or the c-jun promoter.

1 56. The recombinant nucleic acid molecule of claim
2 48 wherein the expression control sequences include cyclic
3 AMP-responsive elements, phorbol ester response elements,
4 serum response element, or Nuclear Factor of Activated T-cells
5 response elements.

1 57. The recombinant nucleic acid molecule of claim
2 48 wherein the expression control sequences are responsive to
3 substances that modulate cell-surface receptors.

1 58. The recombinant nucleic acid molecule of claim
2 48 wherein the expression control sequences are responsive to
3 substances that modulate intra-cellular receptors.

1 59. The recombinant nucleic acid molecule of claim
2 48 wherein the β -lactamase comprises a signal sequence for
3 extracellular secretion.

1 60. The recombinant nucleic acid molecule of claim
2 59 wherein the nucleotide sequence is Sequence 2 of Figure 7.

1 61. The recombinant nucleic acid molecule of claim
2 48 wherein the β -lactamase is a cytosolic β -lactamase.

1 62. The recombinant nucleic acid molecule of claim
2 61 wherein the nucleotide sequence is Sequence 1, Sequence 3,
3 Sequence 4, or Sequence 5 of Fig. 7.

1 63. A recombinant nucleic acid molecule comprising
2 expression control sequences adapted for function in a
3 eukaryotic cell and operably linked to a nucleotide sequence
4 coding for the expression of a cytosolic β -lactamase.

1 64. The recombinant nucleic acid molecule of claim
2 63 wherein the nucleotide sequence encodes a class A β -
3 lactamase.

1 65. The recombinant nucleic acid molecule of claim
2 64 wherein the nucleotide sequence is Sequence 1, Sequence 3,
3 Sequence 4 or Sequence 5 of Figure 7.

1 66. The recombinant nucleic acid molecule of claim
2 63 wherein the expression control sequences are inducible
3 expression control sequences.

1 66. The recombinant nucleic acid molecule of claim
2 63 wherein the expression control sequences are constitutively
3 active expression control sequences.

1 67. The recombinant nucleic acid molecule of claim
2 63 wherein the expression control sequences include the c-fos
3 promoter or the c-jun promoter.

1 68. The recombinant nucleic acid molecule of claim
2 63 wherein the expression control sequences include cyclic
3 AMP-responsive elements, phorbol ester response elements,
4 serum response element, or Nuclear Factor of Activated T-cells
5 response elements.

1 69. The recombinant nucleic acid molecule of claim
2 63 wherein the expression control sequences are responsive to
3 substances that modulate cell-surface receptors.

1 70. The recombinant nucleic acid molecule of claim
2 63 wherein the expression control sequences are responsive to
3 substances that modulate intra-cellular receptors.

1 71. A mammalian host cell transfected with a
2 recombinant nucleic acid molecule further comprising
3 expression control sequences adapted for function in a
4 mammalian cell and operably linked to the nucleotide sequence
5 coding for the expression of a β -lactamase.

1 72. A method of determining the amount of β -
2 lactamase activity in a cell comprising the steps of:
3 providing a host cell transfected with a
4 recombinant nucleic acid molecule comprising expression
5 control sequences operatively linked to nucleic acid sequences
6 coding for the expression of a β -lactamase;

contacting a sample comprising the cell or an extract of the cell or conditioned medium with a substrate for β -lactamase; and

determining the amount of substrate cleaved, whereby the amount of substrate cleaved is related to the amount of β -lactamase activity.

73. A method for monitoring the expression of a gene operably linked to a set of expression control sequences comprising:

providing a eukaryotic host cell transfected with a recombinant nucleic acid molecule comprising expression control sequences operatively linked to nucleic acid sequences coding for the expression of a β -lactamase, except if the eukaryote is a fungus, wherein the β -lactamase is a cytosolic β -lactamase;

contacting a sample comprising the cell or an extract of the cell or conditioned medium with a substrate for β -lactamase; and

determining the amount of substrate cleaved, whereby the amount of substrate cleaved is related to the amount of β -lactamase activity.

74. The method of claim 73 wherein the eukaryotic cell is mammalian.

75. The method of claim 73 wherein the sample comprises an extract from the cell, with or without the conditioned medium.

76. The method of claim 73 wherein the sample comprises the cell.

77. The method of claim 73 wherein the substrate is a compound of claim 1 and the step of determining the amount of substrate cleaved comprises exciting the compound; and determining the degree of fluorescence resonance energy transfer in the sample, whereby a degree of fluorescence

6 resonance energy transfer that is lower than an expected
7 amount indicates the presence of β -lactamase activity.

1 78. The method of claim 73 wherein the nucleotide
2 sequence encodes a class A β -lactamase.

1 79. The method of claim 78 wherein the nucleotide
2 sequence is Sequence 1, Sequence 2, Sequence 3, Sequence 4 or
3 Sequence 5 of Figure 7.

1 80. The method of claim 73 wherein the expression
2 control sequences are inducible expression control sequences.

1 81. The method of claim 73 wherein the cell is an
2 animal cell and the β -lactamase is a cytosolic β -lactamase.

1 82. A method for determining whether a test
2 compound alters the expression of a gene operably linked to a
3 set of expression control sequences comprising:
4 providing a cell transfected with a recombinant
5 nucleic acid construct comprising the expression control
6 sequences operably linked to nucleic acid sequences coding for
7 the expression of a β -lactamase except if the eukaryote is a
8 fungus, wherein the β -lactamase is a cytosolic β -lactamase;
9 contacting the cell with the test compound;
10 contacting a sample comprising the cell or an
11 extract of the cell with a β -lactamase substrate; and
12 determining the amount of substrate cleaved,
13 whereby the amount of substrate cleaved is related to the
14 amount of β -lactamase activity.

1 83. The method of claim 82 wherein the eukaryotic
2 host cell is mammalian.

1 84. The method of claim 82 wherein the sample
2 comprises an extract from the cell.

1 85. The method of claim 82 comprises the cell.

1 86. The method of claim 82 wherein the substrate is
2 a compound of claim 1 and the step of determining the amount
3 of substrate cleaved comprises exciting the compound; and
4 determining the degree of fluorescence resonance energy
5 transfer in the sample, whereby a degree of fluorescence
6 resonance energy transfer that is lower than an expected
7 amount indicates the presence of β -lactamase activity.

1 87. The method of claim 82 wherein the nucleotide
2 sequence encodes a class A β -lactamase.

1 88. The method of claim 87 wherein the nucleotide
2 sequence is Sequence 1, Sequence 2, Sequence 3, Sequence 4 or
3 Sequence 5 of Figure 7.

1 89. The method of claim 85 wherein the expression
2 control sequences are inducible expression control sequences.

1 90. The method of claim 85 wherein the cell is an
2 animal cell and the β -lactamase is a cytosolic β -lactamase.

1 91. The method of claim 82 wherein the expression of
2 β -lactamase is operably regulated by activation, or inhibition
3 of activation, of an intracellular receptor, a cell surface
4 receptor, or a component of an intracellular signaling
5 pathway.

1 92. A method of clonal selection comprising:
2 providing cells transfected with a recombinant
3 nucleic acid molecule comprising the expression control
4 sequences operably linked to nucleic acid sequences coding for
5 the expression of a cytosolic β -lactamase;
6 contacting the cells with a substance that activates
7 or inhibits the activation of the expression control
8 sequences;
9 contacting the cells with a compound of claim 9
10 which is converted into a substrate;

100

11 determining whether substrate is cleaved within each
12 individual cell, whereby cleavage reflects β -lactamase
13 activity;

14 selecting and propagating those cells with a
15 selected level of β -lactamase activity.

16 93. The method of claim 92 further comprising the
17 steps of:

18 culturing selected cells in the absence of
19 activator for a time sufficient for cleaved substrate to be
20 substantially lost from the cells and for β -lactamase levels to
21 return to unactivated levels;

22 incubating the selected cells with a compound
23 of claim 9 which is converted into a substrate; and

24 selecting cells that have not substantially
25 cleaved the substrate.

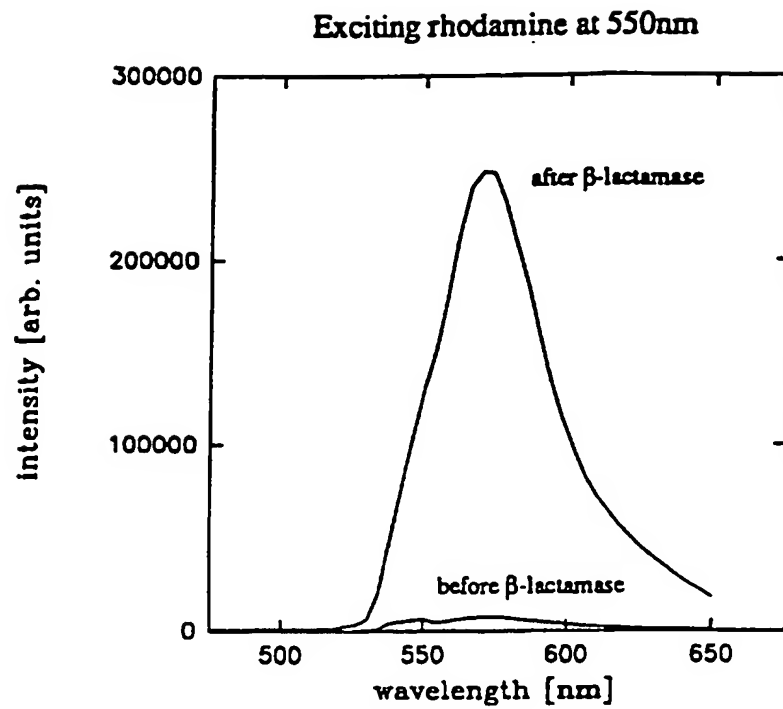


FIG. 1(a)

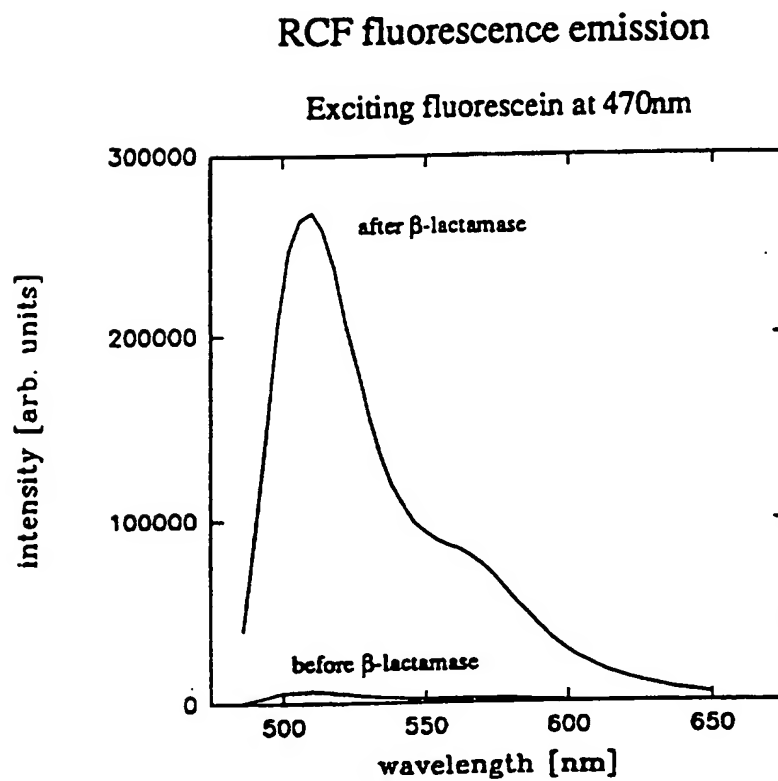
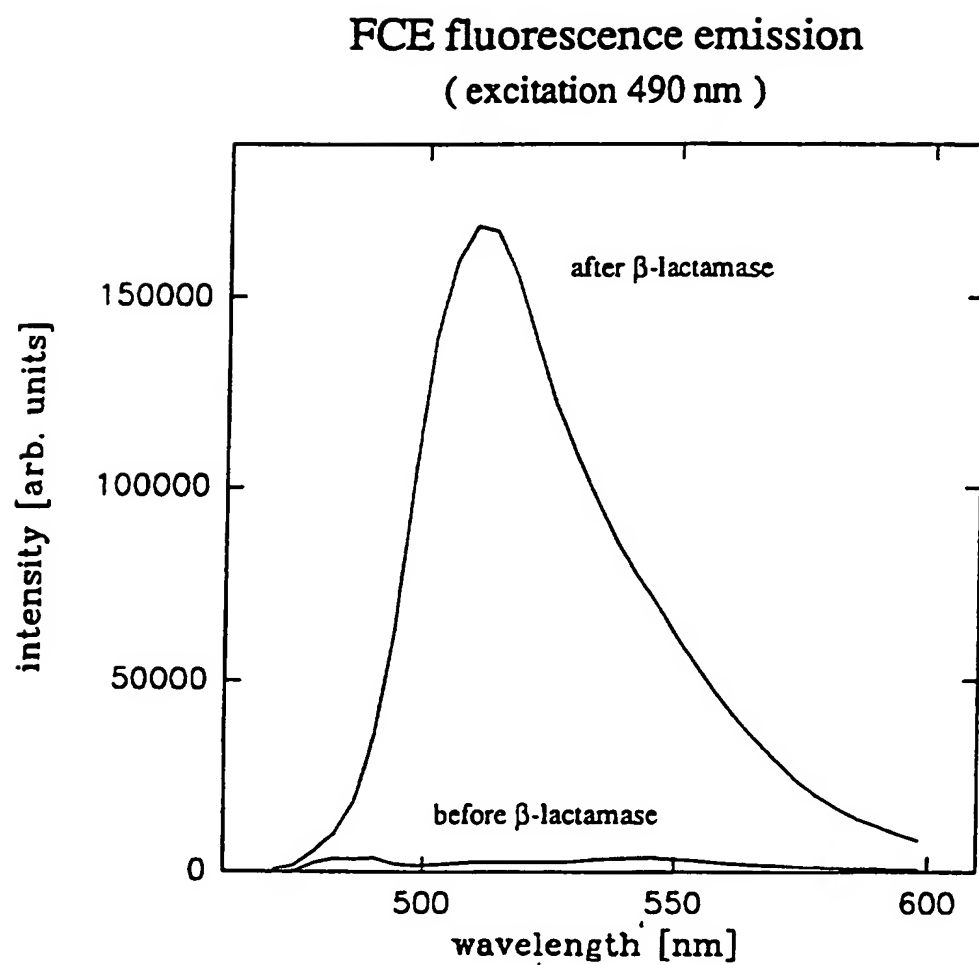


FIG. 1(b)

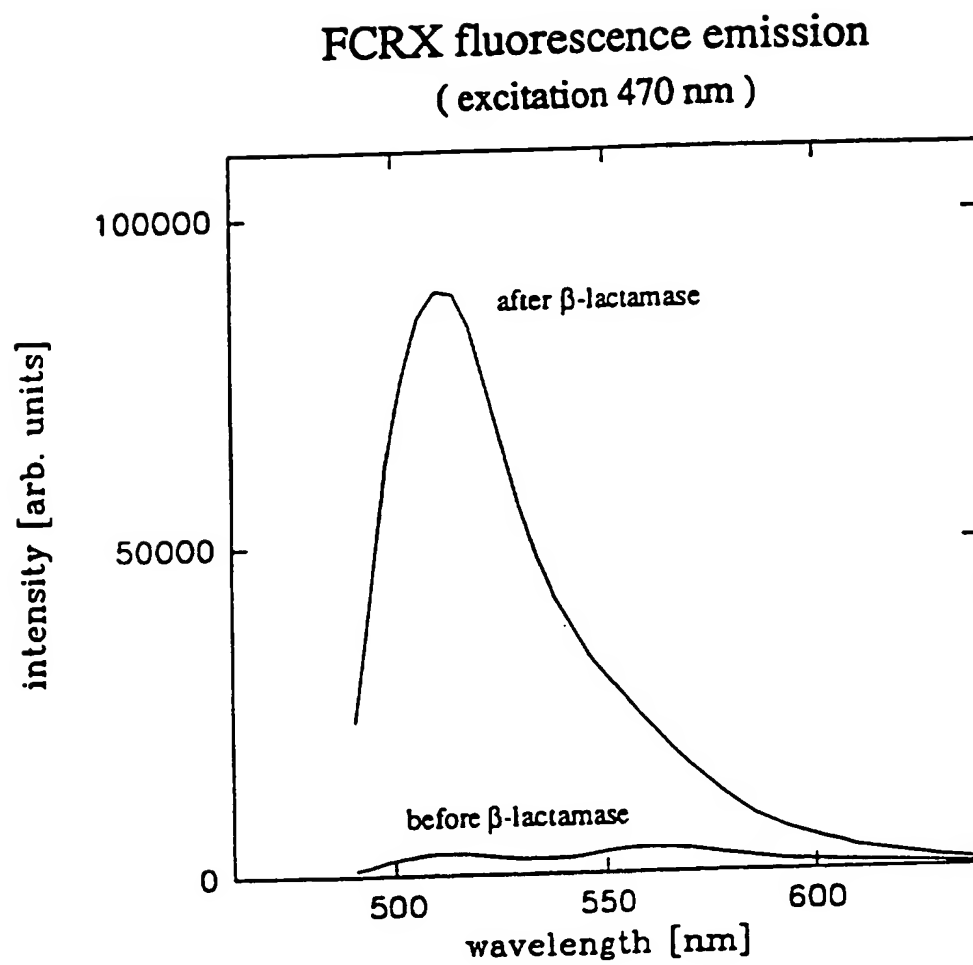
2/17

FIG. 2



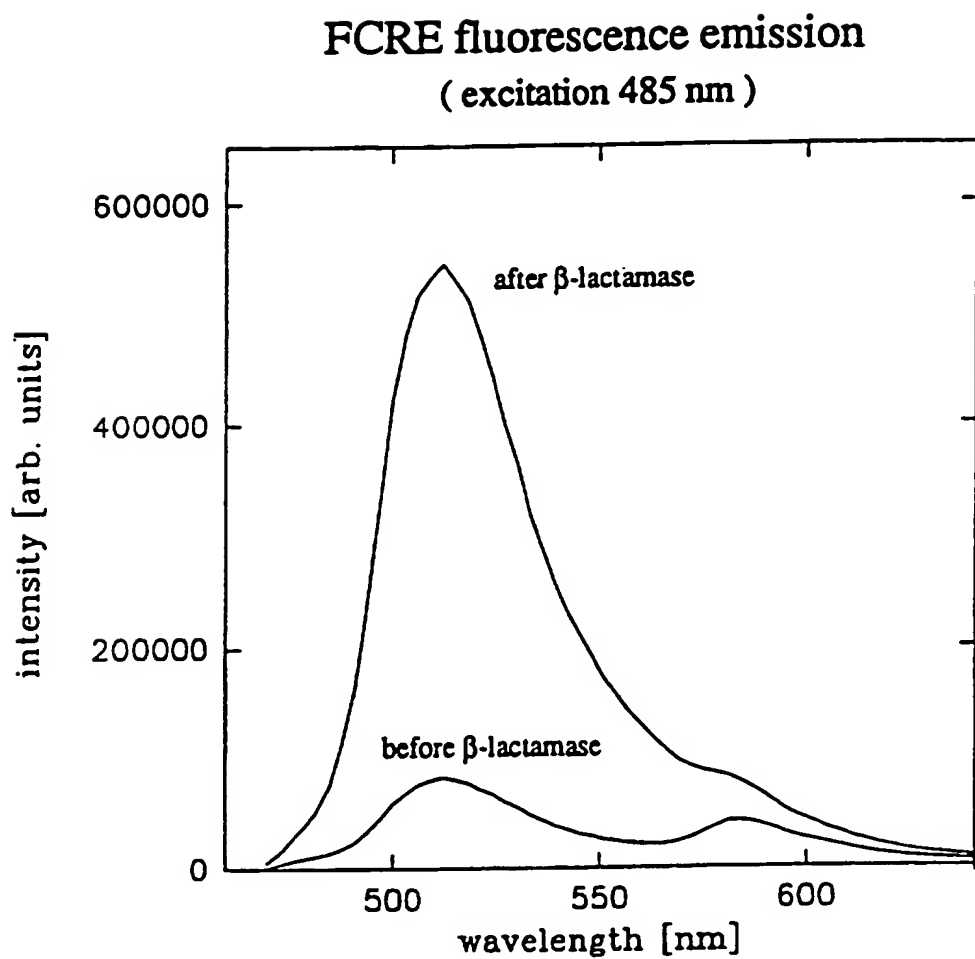
3/17

FIG. 3



4/17

FIG. 4



5/17

Fluorescent β -lactamase substrate (CCF2)

(excitation at 405 nm)

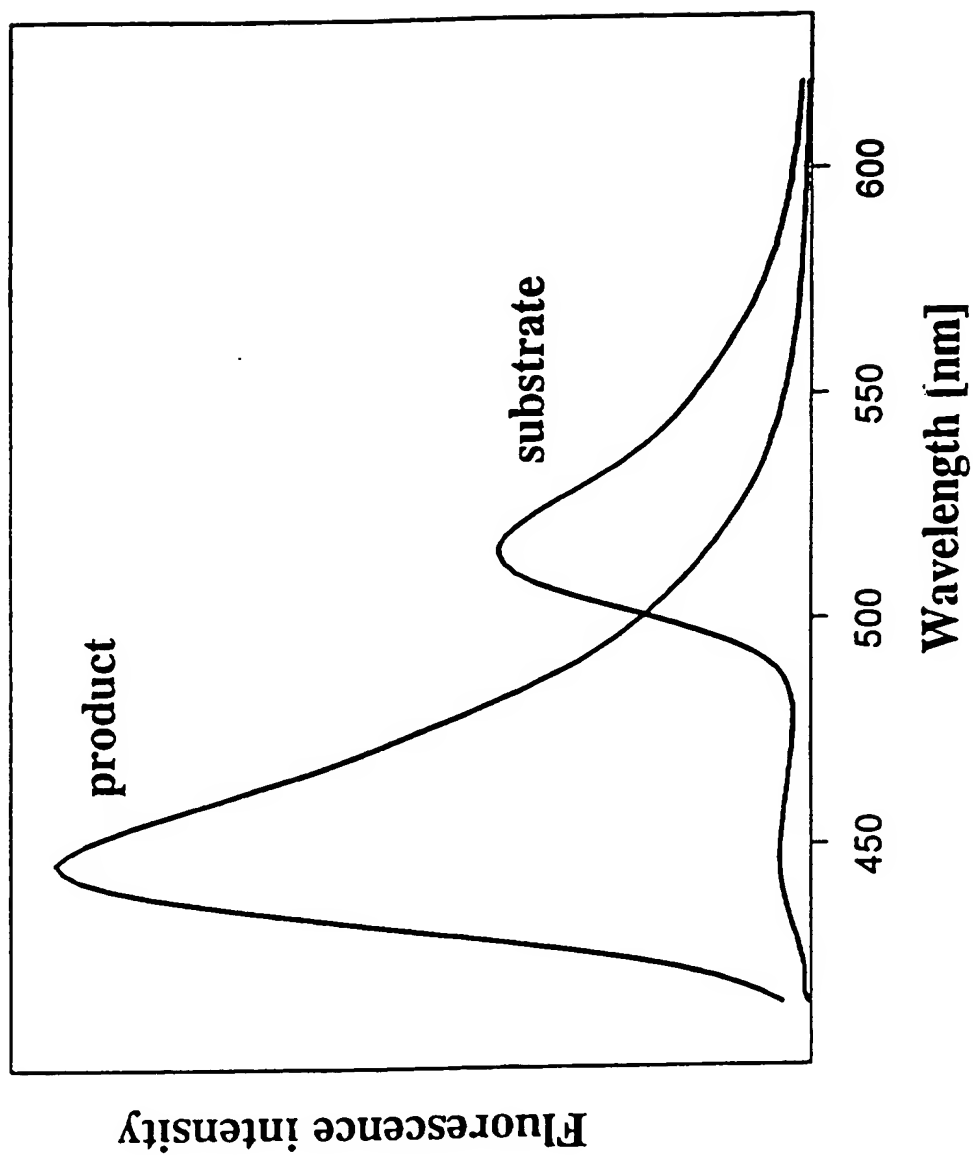


FIG. 5

Fluorescent β -lactamase substrate (CCF1)

(excitation at 390 nm)

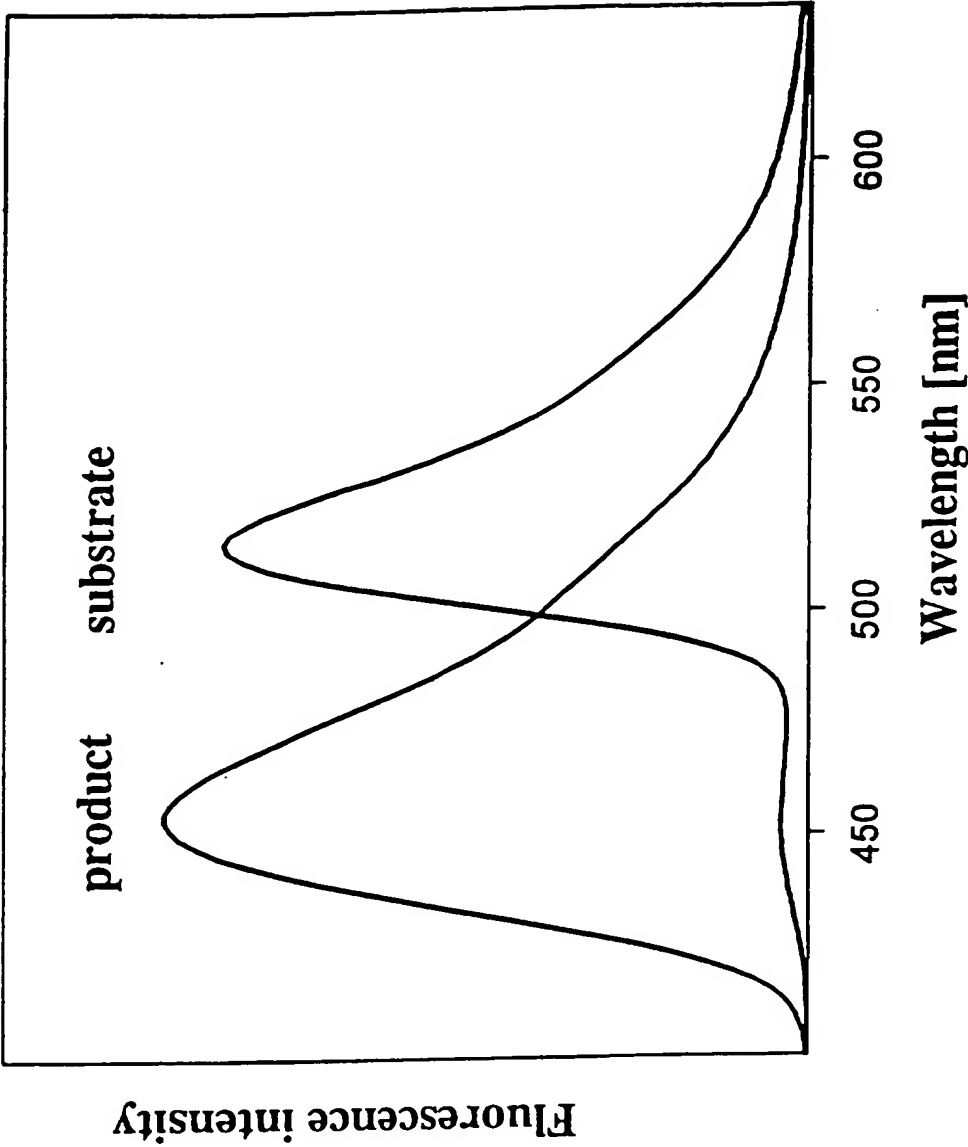


FIG. 6

7/17

FIGURE 7A

Seq ID	parent β -lactamase gene and reference	modification	mammalian expression vector	location of expression
# 1	<i>Escherichia coli</i> RTEM Kadonaga et al.	signal sequence replaced by: ATG TGA	pMAM-neo glucocorticoid-inducible	cytoplasmic
# 2	<i>Escherichia coli</i> RTEM Kadonaga et al.	wild type secreted enzyme 2 changes in pre-sequence: ser 2 arg , ala 23 gly	pMAM-neo glucocorticoid-inducible	secreted extracellularly
# 3	<i>Escherichia coli</i> RTEM	β -globin up stream leader: AAGCTTTTTGCAGAAGCTCA GAATAAACGCAACTTTCCG Kozak sequence: GGTACCACCATGG signal sequence replaced by: ATG GGG	pCDNA 3 CMV promotor and pZEO SV40 promotor	cytoplasmic
# 4	<i>Escherichia coli</i> RTEM	Kozak sequence: GGTACCACCATGG signal sequence replaced by: ATG GAC (GAC replaces CAT)	pMAM-neo glucocorticoid inducible	cytoplasmic
# 5	<i>Bacillus licheniformis</i> 749/C Neugebauer et al.	signal sequence removed, new N-terminal ATG	pCDNA 3 CMV promotor	cytoplasmic

J. Kadonaga et al., *J. Biol. Chem.*, 259: 2149 (1984)K. Neugebauer et al., *Nucleic Acid Res.*, 9: 2577 (1981)

8/17

FIGURE 7B
Sequence no. 1: range 1 to 795
E. coli RTEM as modified by Kadonaga et al (1984)

			10			20			30			40			50	
	*		*		*	*		*	*		*	*		*	*	
ATG	AGT	CAC	CCA	GAA	ACG	CTG	GTG	AAA	GTA	AAA	GAT	GCT	GAA	GAT	CAG	TTG
Met	Ser	His	Pro	Glu	Thr	Leu	Val	Lys	Val	Lys	Asp	Ala	Glu	Asp	Gln	Leu
		60				70			80			90			100	
	*	*		*		*	*		*		*	*		*	*	
GGT	GCA	CGA	GTG	GGT	TAC	ATC	GAA	CTG	GAT	CTC	AAC	AGC	GGT	AAG	ATC	CTT
Gly	Ala	Arg	Val	Gly	Tyr	Ile	Glu	Leu	Asp	Leu	Asn	Ser	Gly	Lys	Ile	Leu
		110			120			130			140				150	
	*	*		*	*		*	*	*		*	*		*	*	
GAG	AGT	TTT	CGC	CCC	GAA	GAA	CGT	TTT	CCA	ATG	ATG	AGC	ACT	TTT	AAA	GTT
Glu	Ser	Phe	Arg	Pro	Glu	Glu	Arg	Phe	Pro	Met	Met	Ser	Thr	Phe	Lys	Val
		160			170			180			190				200	
	*	*	*	*	*	*	*	*	*		*	*	*	*	*	
CTG	CTA	TGT	GGC	GCG	GTA	TTA	TCC	CGT	GTT	GAC	GCC	GGG	CAA	GAG	CAA	CTC
Leu	Leu	Cys	Gly	Ala	Val	Leu	Ser	Arg	Val	Asp	Ala	Gly	Gln	Glu	Gln	Leu
		210			220			230			240				250	
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
GGT	CGC	CGC	ATA	CAC	TAT	TCT	CAG	AAT	GAC	TTG	GTT	GAG	TAC	TCA	CCA	GTC
Gly	Arg	Arg	Ile	His	Tyr	Ser	Gln	Asn	Asp	Leu	Val	Glu	Tyr	Ser	Pro	Val
		260			270			280			290			300		
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
ACA	GAA	AAG	CAT	CTT	ACG	GAT	GGC	ATG	ACA	GTA	AGA	GAA	TTA	TGC	AGT	GCT
Thr	Glu	Lys	His	Leu	Thr	Asp	Gly	Met	Thr	Val	Arg	Glu	Leu	Cys	Ser	Ala
		310			320			330			340			350		
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
GCC	ATA	ACC	ATG	AGT	GAT	AAC	ACT	GCG	GCC	AAC	TTA	CTT	CTG	ACA	ACG	ATC
Ala	Ile	Thr	Met	Ser	Asp	Asn	Thr	Ala	Ala	Asn	Leu	Leu	Leu	Thr	Thr	Ile
			360		370			380			390			400		
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
GGA	GGA	CCG	AAG	GAG	CTA	ACC	GCT	TTT	TTG	CAC	AAC	ATG	GGG	GAT	CAT	GTA
Gly	Gly	Pro	Lys	Glu	Leu	Thr	Ala	Phe	Leu	His	Asn	Met	Gly	Asp	His	Val
			410		420			430			440			450		
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
ACT	CGC	CTT	GAT	CGT	TGG	GAA	CCG	GAG	CTG	AAT	GAA	GCC	ATA	CCA	AAC	GAC
Thr	Arg	Leu	Asp	Arg	Trp	Glu	Pro	Glu	Leu	Asn	Glu	Ala	Ile	Pro	Asn	Asp
			460		470			480			490			500		510
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GAG	CGT	GAC	ACC	ACG	ATG	CCT	GCA	GCA	ATG	GCA	ACA	ACG	TTG	CGC	AAA	CTA
Glu	Arg	Asp	Thr	Thr	Met	Pro	Ala	Ala	Met	Ala	Thr	Thr	Leu	Arg	Lys	Leu

9/17

FIGURE 7C

520		530		540		550		560	
*		*		*		*		*	
TTA	ACT	GGC	GAA	CTA	CTT	ACT	CTA	GCT	TCC
Leu	Thr	Gly	Glu	Leu	Leu	Thr	Leu	Ala	Ser
570				580			590		600
*		*		*		*		*	
ATG	GAG	GCG	GAT	AAA	GTT	GCA	GGA	CCA	CTT
Met	Glu	Ala	Asp	Lys	Val	Ala	Gly	Pro	Leu
620				630			640		650
*		*		*		*		*	
GGC	TGG	TTT	ATT	GCT	GAT	AAA	TCT	GGA	GCC
Gly	Trp	Phe	Ile	Ala	Asp	Lys	Ser	Gly	Ala
670				680			690		700
*		*		*		*		*	
ATC	ATT	GCA	GCA	CTG	GGG	CCA	GAT	GGT	AAG
Ile	Ile	Ala	Ala	Leu	Gly	Pro	Asp	Gly	Lys
720				730			740		750
*		*		*		*		*	
TAC	ACG	ACG	GGG	AGT	CAG	GCA	ACT	ATG	GAT
Tyr	Thr	Thr	Gly	Ser	Gln	Ala	Thr	Met	Asp
770				780			790		
*		*		*		*		*	
GAG	ATA	GGT	GCC	TCA	CTG	ATT	AAG	CAT	TGG
Glu	Ile	Gly	Ala	Ser	Leu	Ile	Lys	His	Trp

10/17

FIGURE 7D

Sequence no. 2: range 1 to 858

Wild-type secreted RTEM enzyme with Ser2→Arg, Ala23→Gly

			10			20			30			40			50	
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
ATG	AGA	ATT	CAA	CAT	TTC	CGT	GTC	GCC	CTT	ATT	CCC	TTT	TTT	GCG	GCA	TTT
Met	Arg	Ile	Gln	His	Phe	Arg	Val	Ala	Leu	Ile	Pro	Phe	Phe	Ala	Ala	Phe
		60				70			80			90				100
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
TGC	CTT	CCT	GTT	TTT	GGT	CAC	CCA	GAA	ACG	CTG	GTG	AAA	GTA	AAA	GAT	GCT
Cys	Leu	Pro	Val	Phe	Gly	His	Pro	Glu	Thr	Leu	Val	Lys	Val	Lys	Asp	Ala
		110				120			130			140				150
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GAA	GAT	CAG	TTG	GGT	GCA	CGA	GTG	GGT	TAC	ATC	GAA	CTG	GAT	CTC	AAC	AGC
Glu	Asp	Gln	Leu	Gly	Ala	Arg	Val	Gly	Tyr	Ile	Glu	Leu	Asp	Leu	Asn	Ser
		160				170			180			190				200
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GGT	AAG	ATC	CTT	GAG	AGT	TTT	CGC	CCC	GAA	GAA	CGT	TTT	CCA	ATG	ATG	AGC
Gly	Lys	Ile	Leu	Glu	Ser	Phe	Arg	Pro	Glu	Glu	Arg	Phe	Pro	Met	Met	Ser
		210				220			230			240				250
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
ACT	TTT	AAA	GTT	CTG	CTA	TGT	GGC	GCG	GTA	TTA	TCC	CGT	GTT	GAC	GCC	GGG
Thr	Phe	Lys	Val	Leu	Leu	Cys	Gly	Ala	Val	Leu	Ser	Arg	Val	Asp	Ala	Gly
		260				270			280			290				300
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CAA	GAG	CAA	CTC	GGT	CGC	CGC	ATA	CAC	TAT	TCT	CAG	AAT	GAC	TTG	GTT	GAG
Gln	Glu	Gln	Leu	Gly	Arg	Arg	Ile	His	Tyr	Ser	Gln	Asn	Asp	Leu	Val	Glu
		310				320			330			340				350
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
TAC	TCA	CCA	GTC	ACA	GAA	AAG	CAT	CTT	ACG	GAT	GGC	ATG	ACA	GTA	AGA	GAA
Tyr	Ser	Pro	Val	Thr	Glu	Lys	His	Leu	Thr	Asp	Gly	Met	Thr	Val	Arg	Glu
		360				370			380			390				400
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
TTA	TGC	AGT	GCT	GCC	ATA	ACC	ATG	AGT	GAT	AAC	ACT	GCG	GCC	AAC	TTA	CTT
Leu	Cys	Ser	Ala	Ala	Ile	Thr	Met	Ser	Asp	Asn	Thr	Ala	Ala	Asn	Leu	Leu
		410				420			430			440				450
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
CTG	ACA	ACG	ATC	GGA	GGA	CCG	AAG	GAG	CTA	ACC	GCT	TTT	TTG	CAC	AAC	ATG
Leu	Thr	Thr	Ile	Gly	Gly	Pro	Lys	Glu	Leu	Thr	Ala	Phe	Leu	His	Asn	Met
		460				470			480			490				510
*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GGG	GAT	CAT	GTA	ACT	CGC	CTT	GAT	CGT	TGG	GAA	CCG	GAG	CTG	AAT	GAA	GCC
Gly	Asp	His	Val	Thr	Arg	Leu	Asp	Arg	Trp	Glu	Pro	Glu	Leu	Asn	Glu	Ala

11/17

FIGURE 7E

			520			530			540			550			560	
	*		*	*		*	*	*	*	*	*	*	*	*	*	
ATA	CCA	AAC	GAC	GAG	CGT	GAC	ACC	ACG	ATG	CCT	GCA	GCA	ATG	GCA	ACA	ACG
Ile	Pro	Asn	Asp	Glu	Arg	Asp	Thr	Thr	Met	Pro	Ala	Ala	Met	Ala	Thr	Thr
		570				580			590			600				610
	*	*		*		*	*	*	*	*	*	*	*	*	*	*
TTG	CGC	AAA	CTA	TTA	ACT	GGC	GAA	CTA	CTT	ACT	CTA	GCT	TCC	CGG	CAA	CAA
Leu	Arg	Lys	Leu	Leu	Thr	Gly	Glu	Leu	Leu	Thr	Leu	Ala	Ser	Arg	Gln	Gln
		620				630			640			650				660
	*	*		*	*	*	*	*	*	*	*	*	*	*	*	*
TTA	ATA	GAC	TGG	ATG	GAG	GCG	GAT	AAA	GTT	GCA	GGA	CCA	CTT	CTG	CGC	TCG
Leu	Ile	Asp	Trp	Met	Glu	Ala	Asp	Lys	Val	Ala	Gly	Pro	Leu	Leu	Arg	Ser
		670				680			690			700				710
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GCC	CTT	CCG	GCT	GGC	TGG	TTT	ATT	GCT	GAT	AAA	TCT	GGA	GCC	GGT	GAG	CGT
Ala	Leu	Pro	Ala	Gly	Trp	Phe	Ile	Ala	Asp	Lys	Ser	Gly	Ala	Gly	Glu	Arg
		720				730			740			750				760
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
GGG	TCT	CGC	GGT	ATC	ATT	GCA	GCA	CTG	GGG	CCA	GAT	GGT	AAG	CCC	TCC	CGT
Gly	Ser	Arg	Gly	Ile	Ile	Ala	Ala	Leu	Gly	Pro	Asp	Gly	Lys	Pro	Ser	Arg
		770				780			790			800				810
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
ATC	GTA	GTT	ATC	TAC	ACG	ACG	GGG	AGT	CAG	GCA	ACT	ATG	GAT	GAA	CGA	AAT
Ile	Val	Val	Ile	Tyr	Thr	Thr	Gly	Ser	Gln	Ala	Thr	Met	Asp	Glu	Arg	Asn
		820				830			840			850				
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AGA	CAG	ATC	GCT	GAG	ATA	GGT	GCC	TCA	CTG	ATT	AAG	CAT	TGG			
Arg	Gln	Ile	Ala	Glu	Ile	Gly	Ala	Ser	Leu	Ile	Lys	His	Trp			

12/17

FIGURE 7F

Sequence no. 3: range 1 to 795

RTEM enzyme with β -globin upstream leader, mammalian Kozak sequence, replacement of signal sequence by Met Gly

```

AAGCTTTTTGCAGAAAGCTCAGAATAAACGCAACTTTCCGGGTACCACC
      *      10      *      20      *      30      *      40      *      50      *
ATG GGG CAC CCA GAA ACG CTG GTG AAA GTA AAA GAT GCT GAA GAT CAG TTG GGT GCA
Met Gly His Pro Glu Thr Leu Val Lys Val Lys Asp Ala Glu Asp Gln Leu Gly Ala
60      70      80      90      100
      *      *      *      *      *      *      *      *      *
CGA GTG GGT TAC ATC GAA CTG GAT CTC AAC AGC GGT AAG ATC CTT GAG AGT
Arg Val Gly Tyr Ile Glu Leu Asp Leu Asn Ser Gly Lys Ile Leu Glu Ser
110      120      130      140      150
      *      *      *      *      *      *      *      *      *
TTT CGC CCC GAA GAA CGT TTT CCA ATG ATG AGC ACT TTT AAA GTT CTG CTA
Phe Arg Pro Glu Glu Arg Phe Pro Met Met Ser Thr Phe Lys Val Leu Leu
160      170      180      190      200      210
      *      *      *      *      *      *      *      *      *
TGT GGC GCG GTA TTA TCC CGT GAT GAC GCC GGG CAA GAG CAA CTC GGT CGC
Cys Gly Ala Val Leu Ser Arg Ile Asp Ala Gly Gln Glu Gln Leu Gly Arg
220      230      240      250      260
      *      *      *      *      *      *      *      *      *
CGC ATA CAC TAT TCT CAG AAT GAC TTG GTT GAG TAC TCA CCA GTC ACA GAA
Arg Ile His Tyr Ser Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr Glu
270      280      290      300      310
      *      *      *      *      *      *      *      *      *
AAG CAT CTT ACG GAT GGC ATG ACA GTA AGA GAA TTA TGC AGT GCT GCC ATA
Lys His Leu Thr Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala Ile
320      330      340      350      360
      *      *      *      *      *      *      *      *      *
ACC ATG AGT GAT AAC ACT GCG GCC AAC TTA CTT CTG ACA ACG ATC GGA GGA
Thr Met Ser Asp Asn Thr Ala Ala Asn Leu Leu Leu Thr Thr Ile Gly Gly
370      380      390      400      410
      *      *      *      *      *      *      *      *      *
CCG AAG GAG CTA ACC GCT TTT TTG CAC AAC ATG GGG GAT CAT GTA ACT CGC
Pro Lys Glu Leu Thr Ala Phe Leu His Asn Met Gly Asp His Val Thr Arg
420      430      440      450      460
      *      *      *      *      *      *      *      *      *
CTT GAT CAT TGG GAA CCG GAG CTG AAT GAA GCC ATA CCA AAC GAC GAG CGT
Leu Asp His Trp Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn Asp Glu Arg

```

FIGURE 7G

[illegible]

14/17

FIGURE 7H

Sequence no. 4: range 1 to 792

RTEM β -lactamase with mammalian Kozak sequence and replacement
of signal sequence by Met Asp

			10			20			30			40			50	
	*		*	*		*	*	*	*	*	*	*	*	*	*	
ATG	GAC	CCA	GAA	ACG	CTG	GTG	AAA	GTA	AAA	GAT	GCT	GAA	GAT	CAG	TTG	GGT
Met	Asp	Pro	Glu	Thr	Leu	Val	Lys	Val	Lys	Asp	Ala	Glu	Asp	Gln	Leu	Gly
		60			70				80			90			100	
	*	*		*		*	*		*		*	*		*		*
GCA	CGA	GTG	GGT	TAC	ATC	GAA	CTG	GAT	CTC	AAC	AGC	GGT	AAG	ATC	CTT	GAG
Ala	Arg	Val	Gly	Tyr	Ile	Glu	Leu	Asp	Leu	Asn	Ser	Gly	Lys	Ile	Leu	Glu
		110			120				130			140			150	
	*	*		*		*	*		*		*	*		*		*
AGT	TTT	CGC	CCC	GAA	GAA	CGT	TTT	CCA	ATG	ATG	AGC	ACT	TTT	AAA	GTT	CTG
Ser	Phe	Arg	Pro	Glu	Glu	Arg	Phe	Pro	Met	Met	Ser	Thr	Phe	Lys	Val	Leu
		160			170				180			190			200	
	*	*		*		*	*		*		*	*		*		*
CTA	TGT	GGC	GCG	GTA	TTA	TCC	CGT	ATT	GAC	GCC	GGG	CAA	GAG	CAA	CTC	GGT
Leu	Cys	Gly	Ala	Val	Leu	Ser	Arg	Ile	Asp	Ala	Gly	Gln	Glu	Gln	Leu	Gly
		210			220				230			240			250	
	*	*		*		*	*		*		*	*		*		*
CGC	CGC	ATA	CAC	TAT	TCT	CAG	AAT	GAC	TTG	GTT	GAG	TAC	TCA	CCA	GTC	ACA
Arg	Arg	Ile	His	Tyr	Ser	Gln	Asn	Asp	Leu	Val	Glu	Tyr	Ser	Pro	Val	Thr
		260			270				280			290			300	
	*	*		*		*	*		*		*	*		*		*
GAA	AAG	CAT	CTT	ACG	GAT	GGC	ATG	ACA	GTA	AGA	GAA	TTA	TGC	AGT	GCT	GCC
Glu	Lys	His	Leu	Thr	Asp	Gly	Met	Thr	Val	Arg	Glu	Leu	Cys	Ser	Ala	Ala
		310			320				330			340			350	
	*	*		*		*	*		*		*	*		*		*
ATA	ACC	ATG	AGT	GAT	AAC	ACT	GCG	GCC	AAC	TTA	CTT	CTG	ACA	ACG	ATC	GGA
Ile	Thr	Met	Ser	Asp	Asn	Thr	Ala	Ala	Asn	Leu	Leu	Leu	Thr	Thr	Ile	Gly
			370				380			390			400			
	*	*		*		*	*		*		*	*		*		*
GGA	CCG	AAG	GAG	CTA	ACC	GCT	TTT	TTG	CAC	AAC	ATG	GGG	GAT	CAT	GTA	ACT
Gly	Pro	Lys	Glu	Leu	Thr	Ala	Phe	Leu	His	Asn	Met	Gly	Asp	His	Val	Thr
			420				430			440			450			
	*	*		*		*	*		*		*	*		*		*
CGC	CTT	GAT	CAT	TGG	GAA	CCG	GAG	CTG	AAT	GAA	GCC	ATA	CCA	AAC	GAC	GAG
Arg	Leu	Asp	Arg	Trp	Glu	Pro	Glu	Leu	Asn	Glu	Ala	Ile	Pro	Asn	Asp	Glu
			470				480			490			500			510
	*	*		*		*	*		*		*	*		*		*
CGT	GAC	ACC	ACG	ATG	CCT	GTA	GCA	ATG	GCA	ACA	ACG	TTG	CGC	AAA	CTA	TTA
Arg	Asp	Thr	Thr	Met	Pro	Val	Ala	Met	Ala	Thr	Thr	Leu	Arg	Lys	Leu	Leu

15/17

FIGURE 7I

			520			530			540			550			560	
	*		*		*	*		*	*		*	*		*	*	
ACT	GGC	GAA	CTA	CTT	ACT	CTA	GCT	TCC	CGG	CAA	CAA	TTA	ATA	GAC	TGG	ATG
Thr	Gly	Glu	Leu	Leu	Thr	Leu	Ala	Ser	Arg	Gln	Gln	Leu	Ile	Asp	Trp	Met
		570				580			590			600			610	
	*	*		*	*	*		*	*		*	*		*	*	*
GAG	GCG	GAT	AAA	GTT	GCA	GGA	CCA	CTT	CTG	CGC	TCG	GCC	CTT	CCG	GCT	GGC
Glu	Ala	Asp	Lys	Val	Ala	Gly	Pro	Leu	Leu	Arg	Ser	Ala	Leu	Pro	Ala	Gly
		620				630			640			650			660	
	*	*		*	*	*		*	*		*	*		*	*	*
TGG	TTT	ATT	GCT	GAT	AAA	TCT	GGA	GCC	GGT	GAG	CGT	GGG	TCT	CGC	GGT	ATC
Trp	Phe	Ile	Ala	Asp	Lys	Ser	Gly	Ala	Gly	Glu	Arg	Gly	Ser	Arg	Gly	Ile
		670				680			690			700			710	
	*	*		*	*	*		*	*		*	*		*	*	*
ATT	GCA	GCA	CTG	GGG	CCA	GAT	GGT	AAG	CCC	TCC	CGT	ATC	GTA	GTT	ATC	TAC
Ile	Ala	Ala	Leu	Gly	Pro	Asp	Gly	Lys	Pro	Ser	Arg	Ile	Val	Val	Ile	Tyr
		720				730			740			750			760	
	*	*		*	*	*		*	*		*	*		*	*	*
ACG	ACG	GGG	AGT	CAG	GCA	ACT	ATG	GAT	GAA	CGA	AAT	AGA	CAG	ATC	GCT	GAG
Thr	Thr	Gly	Ser	Gln	Ala	Thr	Met	Asp	Glu	Arg	Asn	Arg	Gln	Ile	Ala	Glu
		770				780			790							
	*	*		*	*	*		*	*		*	*		*	*	*
ATA	GGT	GCC	TCA	CTG	ATT	AAG	CAT	TGG								
Ile	Gly	Ala	Ser	Leu	Ile	Lys	His	Trp								

16/17

FIGURE 7J

Sequence no. 5: range 1 to 786
Bacillus licheniformis β -lactamase with signal sequence
 replaced

			10				20				30				40			50
	*		*		*		*		*		*		*		*		*	*
ATG	AAA	GAT	GAT	TTT	GCA	AAA	CTT	GAG	GAA	CAA	TTT	GAT	GCA	AAA	CTC	GGG		
Met	Lys	Asp	Asp	Phe	Ala	Lys	Leu	Glu	Glu	Gln	Phe	Asp	Ala	Lys	Leu	Gly		
		60				70			80			90			100			
	*	*		*		*	*		*		*		*		*		*	*
ATC	TTT	GCA	TTG	GAT	ACA	GGT	ACA	AAC	CGG	ACG	GTA	GCG	TAT	CGG	CCG	GAT		
Ile	Phe	Ala	Leu	Asp	Thr	Gly	Thr	Asn	Arg	Thr	Val	Ala	Tyr	Arg	Pro	Asp		
		110				120			130			140			150			
	*	*		*		*	*		*		*		*		*		*	*
GAG	CGT	TTT	GCT	TTT	GCT	TCG	ACG	ATT	AAG	GCT	TTA	ACT	GTA	GGC	GTG	CTT		
Glu	Arg	Phe	Ala	Phe	Ala	Ser	Thr	Ile	Lys	Ala	Leu	Thr	Val	Gly	Val	Leu		
		160				170			180			190			200			
	*	*		*		*	*		*		*		*		*		*	*
TTG	CAA	CAG	AAA	TCA	ATA	GAA	GAT	CTG	AAC	CAG	AGA	ATA	ACA	TAT	ACA	CGT		
Leu	Gln	Gln	Lys	Ser	Ile	Glu	Asp	Leu	Asn	Gln	Arg	Ile	Thr	Tyr	Thr	Arg		
		210				220			230			240			250			
	*	*		*		*	*		*		*		*		*		*	*
GAT	GAT	CTT	GTA	AAC	TAC	AAC	CCG	ATT	ACG	GAA	AAG	CAC	GTT	GAT	ACG	GGA		
Asp	Asp	Leu	Val	Asn	Tyr	Asn	Pro	Ile	Thr	Glu	Lys	His	Val	Asp	Thr	Gly		
		260				270			280			290			300			
	*	*		*		*	*		*		*		*		*		*	*
ATG	ACG	CTC	AAA	GAG	CTT	GCG	GAT	GCT	TCG	CTT	CGA	TAT	AGT	GAC	AAT	GCG		
Met	Thr	Leu	Lys	Glu	Leu	Ala	Asp	Ala	Ser	Leu	Arg	Tyr	Ser	Asp	Asn	Ala		
		310				320			330			340			350			
	*	*		*		*	*		*		*		*		*		*	*
GCA	CAG	AAT	CTC	ATT	CTT	AAA	CAA	ATT	GGC	GGA	CCT	GAA	AGT	TTG	AAA	AAG		
Ala	Gln	Asn	Leu	Ile	Leu	Lys	Gln	Ile	Gly	Gly	Pro	Glu	Ser	Leu	Lys	Lys		
		360				370			380			390			400			
	*	*		*		*	*		*		*		*		*		*	*
GAA	CTG	AGG	AAG	ATT	GGT	GAT	GAG	GTT	ACA	AAT	CCC	GAA	CGA	TTC	GAA	CCA		
Glu	Leu	Arg	Lys	Ile	Gly	Asp	Glu	Val	Thr	Asn	Pro	Glu	Arg	Phe	Glu	Pro		
		410				420			430			440			450			
	*	*		*		*	*		*		*		*		*		*	*
GAG	TTA	AAT	GAA	GTG	AAT	CCG	GGT	GAA	ACT	CAG	GAT	ACC	AGT	ACA	GCA	AGA		
Glu	Leu	Asn	Glu	Val	Asn	Pro	Gly	Glu	Thr	Gln	Asp	Thr	Ser	Thr	Ala	Arg		
		460				470			480			490			500		510	
	*	*		*		*	*		*		*		*		*		*	*
GCA	CTT	GTC	ACA	AGC	CTT	CGA	GCC	TTT	GCT	CTT	GAA	GAT	AAA	CTT	CCA	AGT		
Ala	Leu	Val	Thr	Ser	Leu	Arg	Ala	Phe	Ala	Leu	Glu	Asp	Lys	Leu	Pro	Ser		

FIGURE 7K

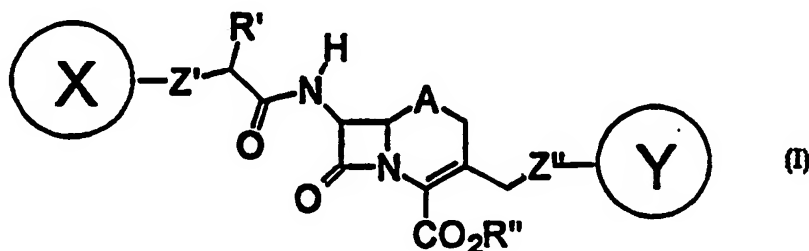
[illegible]



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07D 471/04, 491/04, 501/18, C12Q 1/00		A3	(11) International Publication Number: WO 96/30540
			(43) International Publication Date: 3 October 1996 (03.10.96)
(21) International Application Number: PCT/US96/04059		(74) Agent: STORELLA, John, R.; Townsend and Townsend and Crew, Steuart Street Tower, 20th floor, One Market Plaza, San Francisco, CA 94105 (US).	
(22) International Filing Date: 20 March 1996 (20.03.96)			
(30) Priority Data: 08/407,544 20 March 1995 (20.03.95) US		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(60) Parent Application or Grant (63) Related by Continuation US Filed on 08/407,544 (CIP) 20 March 1995 (20.03.95)			
(71) Applicant (for all designated States except US): THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612 (US).		Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
(72) Inventors; and (75) Inventors/Applicants (for US only): TSIEN, Roger, Y. [US/US]; 8535 Nottingham Place, La Jolla, CA 92037 (US). ZLOKARNIK, Gregor [DE/US]; 3329 Jemez Drive, San Diego, CA 92117 (US).		(88) Date of publication of the international search report: 9 January 1997 (09.01.97)	

(54) Title: SUBSTRATES FOR BETA-LACTAMASE AND USES THEREOF



(57) Abstract

Substrates for β -lactamase of general formula (I) in which one of X and Y is a fluorescent donor moiety and the other is a quencher (which may or may not re-emit); R' is selected from the group consisting of H, lower (i.e., alkyl of 1 to about 5 carbon atoms) and $(CH_2)_nOH$, in which n is 0 or an integer from 1 to 5; R'' is selected from the group consisting of H, physiologically acceptable metal and ammonium cations, $-CHR^2OCO(CH_2)_nCH_3$, $-CHR^2OCOC(CH_3)_3$, acylthiomethyl, acyloxy-alpha-benzyl, delta-butyrolactonyl, methoxycarbonyloxymethyl, phenyl, methylsulphinylmethyl, beta-morpholinoethyl, dialkylaminoethyl, acyloxyalkyl, dialkylaminocarbonyloxymethyl and aliphatic, in which R² is selected from the group consisting of H and lower alkyl; A is selected from the group consisting of S, O, SO, SO₂ and CH₂; and Z' and Z'' are linkers for the fluorescent donor and quencher moieties. Methods of assaying β -lactamase activity and monitoring expression in systems using β -lactamase as a reporter gene also are disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

BEST AVAILABLE COPY

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/04059

A. CLASSIFICATION OF SUBJECT MATTER

C 07 D 471/04, C 07 D 491/04, C 07D 501/18, C 12 Q 1/00

According to International Patent Classification (IPC) or to both national classification and IPC 6

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C 07 D, C 12 Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No..
A	EP, A, 0 354 757 (BEECHAM GROUP PLC) 14 February 1990 (14.02.90), claims 1-10.	1-93
A	US, A, 5 162 524 (FARINA et al.) 10 November 1992 (10.11.92), claims 1-12.	1-93
A	EP, A, 0 053 541 (RHONE-POULENC IND.) 09 June 1982 (09.06.82), claims 1-4.	1-93



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- * "A" document defining the general state of the art which is not considered to be of particular relevance
- * "E" earlier document but published on or after the international filing date
- * "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- * "O" document referring to an oral disclosure, use, exhibition or other means
- * "P" document published prior to the international filing date but later than the priority date claimed

- * "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- * "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- * "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- * "&" document member of the same patent family

Date of the actual completion of the international search

17 October 1996

Date of mailing of the international search report

05.11.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

BRUS e.h.

BEST AVAILABLE COPY

zum internationalen Recherchen-
bericht über die internationale
Patentmeldung Nr.

to the International Search
Report to the International Patent
Application No.

au rapport de recherche inter-
national relatif à la demande de brevet
international n°

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentedokumente angegeben. Diese Angaben dienen nur zur Orientierung und erfolgen ohne Gewähr.

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The Office is in no way liable for these particulars which are given merely for the purpose of information.

La présente annexe indique les membres de la famille de brevets relatifs aux documents de brevets cités dans le rapport de recherche international visée ci-dessus. Les renseignements fournis sont données à titre indicatif et n'engagent pas la responsabilité de l'Office.

Ia Recherchenbericht angeführtes Patentedokument Patent document cited in search report Document de brevet cité dans le rapport de recherche		Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
EP A2	354757	14-02-90	EP A3 354757 JP A0 19104 JP A2 2041073	29-05-91 14-09-90 30-03-90
US A	5162524	10-11-92	US A 5266691	30-11-93
EP A1	53541	09-06-82	AT E 88005 AT F 88005 AT G 88005 AT H 88005 AT I 88005 AT J 88005 AT K 88005 AT L 88005 AT M 88005 AT N 88005 AT O 88005 AT P 88005 AT Q 88005 AT R 88005 AT S 88005 AT T 88005 AT U 88005 AT V 88005 AT W 88005 AT X 88005 AT Y 88005 AT Z 88005 AT AA 88005 AT AB 88005 AT AC 88005 AT AD 88005 AT AE 88005 AT AF 88005 AT AG 88005 AT AH 88005 AT AI 88005 AT AJ 88005 AT AK 88005 AT AL 88005 AT AM 88005 AT AN 88005 AT AO 88005 AT AP 88005 AT AQ 88005 AT AR 88005 AT AS 88005 AT AT 88005 AT AU 88005 AT AV 88005 AT AW 88005 AT AX 88005 AT AY 88005 AT AZ 88005 AT BA 88005 AT BB 88005 AT BC 88005 AT BD 88005 AT BE 88005 AT BF 88005 AT BG 88005 AT BH 88005 AT BI 88005 AT BJ 88005 AT BK 88005 AT BL 88005 AT BM 88005 AT BN 88005 AT BO 88005 AT BP 88005 AT BQ 88005 AT BR 88005 AT BS 88005 AT BT 88005 AT BU 88005 AT BV 88005 AT BW 88005 AT BX 88005 AT BY 88005 AT BZ 88005 AT CA 88005 AT CB 88005 AT CC 88005 AT CD 88005 AT CE 88005 AT CF 88005 AT CG 88005 AT CH 88005 AT CI 88005 AT CJ 88005 AT CK 88005 AT CL 88005 AT CM 88005 AT CN 88005 AT CO 88005 AT CP 88005 AT CQ 88005 AT CR 88005 AT CS 88005 AT CT 88005 AT CU 88005 AT CV 88005 AT CW 88005 AT CX 88005 AT CY 88005 AT CZ 88005 AT DA 88005 AT DB 88005 AT DC 88005 AT DD 88005 AT DE 88005 AT DF 88005 AT DG 88005 AT DH 88005 AT DI 88005 AT DJ 88005 AT DK 88005 AT DL 88005 AT DM 88005 AT DN 88005 AT DO 88005 AT DP 88005 AT DQ 88005 AT DR 88005 AT DS 88005 AT DT 88005 AT DU 88005 AT DV 88005 AT DW 88005 AT DX 88005 AT DY 88005 AT DZ 88005 AT EA 88005 AT EB 88005 AT EC 88005 AT ED 88005 AT EE 88005 AT EF 88005 AT EG 88005 AT EH 88005 AT EI 88005 AT EJ 88005 AT EK 88005 AT EL 88005 AT EM 88005 AT EN 88005 AT EO 88005 AT EP 88005 AT EQ 88005 AT ER 88005 AT ES 88005 AT ET 88005 AT EU 88005 AT EV 88005 AT EW 88005 AT EX 88005 AT EY 88005 AT EZ 88005 AT FA 88005 AT FB 88005 AT FC 88005 AT FD 88005 AT FE 88005 AT FF 88005 AT FG 88005 AT FH 88005 AT FI 88005 AT FJ 88005 AT FK 88005 AT FL 88005 AT FM 88005 AT FN 88005 AT FO 88005 AT FP 88005 AT FQ 88005 AT FR 88005 AT FS 88005 AT FT 88005 AT FU 88005 AT FV 88005 AT FW 88005 AT FX 88005 AT FY 88005 AT FZ 88005 AT GA 88005 AT GB 88005 AT GC 88005 AT GD 88005 AT GE 88005 AT GF 88005 AT GG 88005 AT GH 88005 AT GI 88005 AT GJ 88005 AT GK 88005 AT GL 88005 AT GM 88005 AT GN 88005 AT GO 88005 AT GP 88005 AT GQ 88005 AT GR 88005 AT GS 88005 AT GT 88005 AT GU 88005 AT GV 88005 AT GW 88005 AT GX 88005 AT GY 88005 AT GZ 88005 AT HA 88005 AT HB 88005 AT HC 88005 AT HD 88005 AT HE 88005 AT HF 88005 AT HG 88005 AT HH 88005 AT HI 88005 AT HJ 88005 AT HK 88005 AT HL 88005 AT HM 88005 AT HN 88005 AT HO 88005 AT HP 88005 AT HQ 88005 AT HR 88005 AT HS 88005 AT HT 88005 AT HU 88005 AT HV 88005 AT HW 88005 AT HX 88005 AT HY 88005 AT HZ 88005 AT IA 88005 AT IB 88005 AT IC 88005 AT ID 88005 AT IE 88005 AT IF 88005 AT IG 88005 AT IH 88005 AT II 88005 AT IJ 88005 AT IK 88005 AT IL 88005 AT IM 88005 AT IN 88005 AT IO 88005 AT IP 88005 AT IQ 88005 AT IR 88005 AT IS 88005 AT IT 88005 AT IU 88005 AT IV 88005 AT IW 88005 AT IX 88005 AT IY 88005 AT IZ 88005 AT JA 88005 AT JB 88005 AT JC 88005 AT JD 88005 AT JE 88005 AT JF 88005 AT JG 88005 AT JH 88005 AT JI 88005 AT JJ 88005 AT JK 88005 AT JL 88005 AT JM 88005 AT JN 88005 AT JO 88005 AT JP 88005 AT JQ 88005 AT JR 88005 AT JS 88005 AT JT 88005 AT JU 88005 AT JV 88005 AT JW 88005 AT JX 88005 AT JY 88005 AT JZ 88005 AT KA 88005 AT KB 88005 AT KC 88005 AT KD 88005 AT KE 88005 AT KF 88005 AT KG 88005 AT KH 88005 AT KI 88005 AT KJ 88005 AT KK 88005 AT KL 88005 AT KM 88005 AT KN 88005 AT KO 88005 AT KP 88005 AT KQ 88005 AT KR 88005 AT KS 88005 AT KT 88005 AT KU 88005 AT KV 88005 AT KW 88005 AT KX 88005 AT KY 88005 AT KZ 88005 AT LA 88005 AT LB 88005 AT LC 88005 AT LD 88005 AT LE 88005 AT LF 88005 AT LG 88005 AT LH 88005 AT LI 88005 AT LJ 88005 AT LK 88005 AT LL 88005 AT LM 88005 AT LN 88005 AT LO 88005 AT LP 88005 AT LQ 88005 AT LR 88005 AT LS 88005 AT LT 88005 AT LU 88005 AT LV 88005 AT LW 88005 AT LX 88005 AT LY 88005 AT LZ 88005 AT MA 88005 AT MB 88005 AT MC 88005 AT MD 88005 AT ME 88005 AT MF 88005 AT MG 88005 AT MH 88005 AT MI 88005 AT MJ 88005 AT MK 88005 AT ML 88005 AT MM 88005 AT MN 88005 AT MO 88005 AT MP 88005 AT MQ 88005 AT MR 88005 AT MS 88005 AT MT 88005 AT MU 88005 AT MV 88005 AT MW 88005 AT MX 88005 AT MY 88005 AT MZ 88005 AT NA 88005 AT NB 88005 AT NC 88005 AT ND 88005 AT NE 88005 AT NF 88005 AT NG 88005 AT NH 88005 AT NI 88005 AT NJ 88005 AT NK 88005 AT NL 88005 AT NM 88005 AT NO 88005 AT NP 88005 AT NQ 88005 AT NR 88005 AT NS 88005 AT NT 88005 AT NU 88005 AT NV 88005 AT NW 88005 AT NX 88005 AT NY 88005 AT NZ 88005 AT OA 88005 AT OB 88005 AT OC 88005 AT OD 88005 AT OE 88005 AT OF 88005 AT OG 88005 AT OH 88005 AT OI 88005 AT OJ 88005 AT OK 88005 AT OL 88005 AT OM 88005 AT ON 88005 AT OO 88005 AT OP 88005 AT OQ 88005 AT OR 88005 AT OS 88005 AT OT 88005 AT OU 88005 AT OV 88005 AT OW 88005 AT OX 88005 AT OY 88005 AT OZ 88005 AT PA 88005 AT PB 88005 AT PC 88005 AT PD 88005 AT PE 88005 AT PF 88005 AT PG 88005 AT PH 88005 AT PI 88005 AT PJ 88005 AT PK 88005 AT PL 88005 AT PM 88005 AT PN 88005 AT PO 88005 AT PP 88005 AT PQ 88005 AT PR 88005 AT PS 88005 AT PT 88005 AT PU 88005 AT PV 88005 AT PW 88005 AT PX 88005 AT PY 88005 AT PZ 88005 AT QA 88005 AT QB 88005 AT QC 88005 AT QD 88005 AT QE 88005 AT QF 88005 AT QG 88005 AT QH 88005 AT QI 88005 AT QJ 88005 AT QK 88005 AT QL 88005 AT QM 88005 AT QN 88005 AT QO 88005 AT QP 88005 AT QQ 88005 AT QR 88005 AT QS 88005 AT QT 88005 AT QU 88005 AT QV 88005 AT QW 88005 AT QX 88005 AT QY 88005 AT QZ 88005 AT RA 88005 AT RB 88005 AT RC 88005 AT RD 88005 AT RE 88005 AT RF 88005 AT RG 88005 AT RH 88005 AT RI 88005 AT RJ 88005 AT RK 88005 AT RL 88005 AT RM 88005 AT RN 88005 AT RO 88005 AT RP 88005 AT RQ 88005 AT RR 88005 AT RS 88005 AT RT 88005 AT RU 88005 AT RV 88005 AT RW 88005 AT RX 88005 AT RY 88005 AT RZ 88005 AT SA 88005 AT SB 88005 AT SC 88005 AT SD 88005 AT SE 88005 AT SF 88005 AT SG 88005 AT SH 88005 AT SI 88005 AT SJ 88005 AT SK 88005 AT SL 88005 AT SM 88005 AT SN 88005 AT SO 88005 AT SP 88005 AT SQ 88005 AT SR 88005 AT SS 88005 AT ST 88005 AT SU 88005 AT SV 88005 AT SW 88005 AT SX 88005 AT SY 88005 AT SZ 88005 AT TA 88005 AT TB 88005 AT TC 88005 AT TD 88005 AT TE 88005 AT TF 88005 AT TG 88005 AT TH 88005 AT TI 88005 AT TJ 88005 AT TK 88005 AT TL 88005 AT TM 88005 AT TN 88005 AT TO 88005 AT TP 88005 AT TQ 88005 AT TR 88005 AT TS 88005 AT TT 88005 AT TU 88005 AT TV 88005 AT TW 88005 AT TX 88005 AT TY 88005 AT TZ 88005 AT UA 88005 AT UB 88005 AT UC 88005 AT UD 88005 AT UE 88005 AT UF 88005 AT UG 88005 AT UH 88005 AT UI 88005 AT UJ 88005 AT UK 88005 AT UL 88005 AT UM 88005 AT UN 88005 AT UO 88005 AT UP 88005 AT UQ 88005 AT UR 88005 AT US 88005 AT UT 88005 AT UV 88005 AT UW 88005 AT UX 88005 AT UY 88005 AT UZ 88005 AT VA 88005 AT VB 88005 AT VC 88005 AT VD 88005 AT VE 88005 AT VF 88005 AT VG 88005 AT VH 88005 AT VI 88005 AT VJ 88005 AT VK 88005 AT VL 88005 AT VM 88005 AT VN 88005 AT VO 88005 AT VP 88005 AT VQ 88005 AT VR 88005 AT VS 88005 AT VT 88005 AT VU 88005 AT VV 88005 AT VW 88005 AT VX 88005 AT VY 88005 AT VZ 88005 AT WA 88005 AT WB 88005 AT WC 88005 AT WD 88005 AT WE 88005 AT WF 88005 AT WG 88005 AT WH 88005 AT WI 88005 AT WJ 88005 AT WK 88005 AT WL 88005 AT WM 88005 AT WN 88005 AT WO 88005 AT WP 88005 AT WQ 88005 AT WR 88005 AT WS 88005 AT WT 88005 AT WU 88005 AT WV 88005 AT WW 88005 AT WX 88005 AT WY 88005 AT WZ 88005 AT XA 88005 AT XB 88005 AT XC 88005 AT XD 88005 AT XE 88005 AT XF 88005 AT XG 88005 AT XH 88005 AT XI 88005 AT XJ 88005 AT XK 88005 AT XL 88005 AT XM 88005 AT XN 88005 AT XO 88005 AT XP 88005 AT XQ 88005 AT XR 88005 AT XS 88005 AT XT 88005 AT XU 88005 AT XV 88005 AT XW 88005 AT XX 88005 AT XY 88005 AT XZ 88005 AT YA 88005 AT YB 88005 AT YC 88005 AT YD 88005 AT YE 88005 AT YF 88005 AT YG 88005 AT YH 88005 AT YI 88005 AT YJ 88005 AT YK 88005 AT YL 88005 AT YM 88005 AT YN 88005 AT YO 88005 AT YP 88005 AT YQ 88005 AT YR 88005 AT YS 88005 AT YT 88005 AT YU 88005 AT YV 88005 AT YW 88005 AT YX 88005 AT YY 88005 AT YZ 88005 AT ZA 88005 AT ZB 88005 AT ZC 88005 AT ZD 88005 AT ZE 88005 AT ZF 88005 AT ZG 88005 AT ZH 88005 AT ZI 88005 AT ZJ 88005 AT ZK 88005 AT ZL 88005 AT ZM 88005 AT ZN 88005 AT ZO 88005 AT ZP 88005 AT ZQ 88005 AT ZR 88005 AT ZS 88005 AT ZT 88005 AT ZU 88005 AT ZV 88005 AT ZW 88005 AT ZX 88005 AT ZY 88005 AT ZZ 88005	

BEST AVAILABLE COPY